LICOFELONE, A BALANCED INHIBITOR OF CYCLOOXYGENASE AND 5-LIPOXYGENASE, REDUCES INFLAMMATION IN A RABBIT MODEL OF Atherosclerosis

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Abbreviations: 5-LOX, 5-lipoxygenase; COX-2, cyclooxygenase-2; COX-1, cyclooxygenase-1; LIC, licofelone; ROF, rofecoxib, NT, non treated; MCP-1, monocyte chemoattractant protein 1; NF-kB, nuclear factor-kB; LTB4, leukotriene B4; TXB2, thromboxane B2; PGE2, prostaglandin E2; VSMC, Vascular Smooth Muscle cells; BLT1, leukotriene B4 receptor 1; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α.

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ABSTRACT
Licofelone, a dual anti-inflammatory drug, which inhibits 5-lipoxygenase and cyclooxygenase enzymes, may have a better cardiovascular profile that cyclooxygenase-2 inhibitors due to cyclooxygenase-1 blockade mediated antithrombotic effect and a better gastrointestinal tolerability. We examined the anti-inflammatory effect of licofelone on atherosclerotic lesions as well as in isolated neutrophils from whole blood of rabbits compared to a selective inhibitor of COX-2, rofecoxib. We also assessed the antithrombotic effect of licofelone in rabbit platelet-rich plasma. For this purpose, thirty rabbits underwent injury of femoral arteries and were randomized to receive 10 mg/kg/day of licofelone or 5 mg/kg/day of rofecoxib or no treatment during 4 weeks with atherogenic diet in all cases. Ten healthy rabbits were used as controls. Neutrophils and platelets were isolated from peripheral blood of rabbits for ex vivo studies. Licofelone reduced intima/media ratio in injured arteries, the macrophages infiltration in the neointimal area, MCP-1 gene expression and the activation of NF-κB in rabbit atheroma. Moreover, licofelone inhibited COX-2 and 5-LOX protein expression in vascular lesions. Rofecoxib only diminished COX-2 protein expression and MCP-1 gene expression in vascular atheroma. PGE2 in rabbit plasma was attenuated by both drugs. Licofelone almost abolished 5-LOX activity by inhibiting LTB4 generation in rabbit neutrophils and prevented platelet TXB2 production from whole blood. Licofelone reduces neointimal formation and inflammation in an atherosclerotic rabbit model more markedly than rofecoxib. This effect, together with the antiplatelet activity of licofelone, suggests that this drug may have a favorable cardiovascular profile.
INTRODUCTION

Atherosclerosis is an inflammatory disease that involves the recruitment of monocytes into the arterial wall, a process driven by adhesion molecules and chemoattractant cytokines, mainly MCP-1 (monocyte chemoattractant protein-1) (Hernández-Presa et al., 1997). These inflammatory cells express metalloproteinases that weaken the fibrous cap of the lesions making them prone to rupture and subsequent thrombosis, causing an acute coronary syndrome (Nomoto et al., 2004).

COX-2 is expressed mainly in inflammatory disorders, such as atherosclerosis, and converts arachidonic acid to PGG₂ (Linton et al., 2004). Depending on the enzymes working downstream, PGG₂ may yield a variety of PGs and TXA₂, with opposite effects on vascular wall biology. COX-2 is co-expressed frequently with PGE synthase, yielding PGE₂ that possesses proinflammatory and chemoattractant properties. Then, theoretically, COX-2 blockade could be beneficial in atherothrombosis. However, clinical trials have shown different viewpoints about the protector or hazardous effect of selective COX-2 inhibitors.

Given that TXA₂ is produced in platelets via COX-1, selective COX-2 inhibition may leave this pathway unblocked. Therefore, it has been suggested that COX-2 inhibitors would lead to an imbalance between prothrombotic TXA₂ and vasodilatory prostacyclines (PGI₂), which may be synthesized via COX-2, favoring platelet aggregation and vasoconstriction.

In the VIGOR study, the COX-2 inhibitor rofecoxib increased the risk of cardiovascular events in patients with rheumatoid arthritis (Bombardier et al., 2000). Although in a similar study celecoxib did not increase the cardiovascular risk (Silverstein et al., 2000), and even some favorable effects were reported with this drug and with meloxicam (Altman et al., 2002; Chevenerd et al., 2003) recent data from patients with colon adenoma suggest that both rofecoxib and celecoxib may increase cardiovascular risk at long-term (Bresalier et al., 2005; Solomon et al., 2005). As a result, the clinical evidence of an increased risk of cardiovascular events in patients taking COX-2 inhibitors remains controversial (Mukherjee et al., 2001).
5-lipoxygenase (5-LOX) is another enzyme involved in the metabolism of arachidonic acid that catalyses leukotriene (LT) production, inflammation, apoptosis, proliferation and atherogenesis (Spanbroek et al., 2003). 5-LOX is expressed in monocytes and macrophages, and contributes to the development and rupture of atherosclerotic plaques (Steinhilber, 1999). Polymorphisms in the 5-LOX gene promoter and certain 5-LOX-activating protein (FLAP) haplotypes have been linked to an increased risk of infarction and stroke (Zhao et al., 2004).

In the last years, several new 5-LOX and COX-2 inhibitors have been developed (Martel-Pelletier et al., 2003) in a search for an anti-inflammatory compound with higher gastrointestinal safety than the classical non-steroidal anti-inflammatory drugs and without the cardiovascular risk associated to COX-2 inhibitors. Licofelone is one of these potent anti-inflammatory drugs, which inhibits COX-2, COX-1 and 5-LOX. In in vitro studies, licofelone has been reported to suppress both 5-LOX (with an IC50 of 0.18µM) and COX-2 (with an IC50 of 0.21µM) (Singh et al., 2005).

Licofelone has shown anti-inflammatory, analgesic and antiasthmatic effects in several experimental models at dosages between 10 and 100mg/Kg that do not cause any gastrointestinal damage (Rotondo et al., 2002). The anti-inflammatory action of this drug has been shown in animal models of osteoarthritis (Jovanovic et al., 2004; Pelletier et al., 2005), and it is currently being evaluated in phase III clinical trials in this disorder. Moreover, antithrombotic effects of licofelone due to inhibition of COX-1-mediated platelet function have been reported in mice and rat models (Tries et al., 2002) and in human platelets (Rotondo et al., 2004). Licofelone, compared to classical NSAIDs, have been found to possess a unique ability to inhibit leukocyte rolling and adhesion to endothelium (Ulbrich et al., 2005). Its analgesic, anti-inflammatory and antiplatelet properties are present at doses, which are safe for gastrointestinal tract (Cicero et al., 2005). The results from a ramdomized trial in healthy human volunteers indicate that licofelone has a potential gastrointestinal safety advantage over conventional NSAID therapy, as licofelone (200 or 400 mg b.i.d) was associated with a lower incidence of ulcers compared with naproxeno (500 mg b.i.d) (Bias et al., 2004).
Consequently, since licofelone shares the anti-inflammatory effect and gastric safety of COX-2 inhibitors (Lehmann et al., 2005), but also inhibits COX-1-mediated platelet function, therefore avoiding the prothrombotic state, this drug may have a better cardiovascular profile than COX-2 inhibitors. The aim of this study was to investigate the effects of licofelone, in comparison to the selective COX-2 inhibitor rofecoxib, on the inflammatory response in vascular lesions in a rabbit atherosclerosis model as well as in rabbit neutrophils and platelet-rich plasma isolated from whole blood. Additional in vitro studies were also performed in vascular smooth muscle cells (VSMC).
METHODS

Studies in the experimental model

Experimental model

Forty New Zealand male rabbits (3.5–4 kg) were used. The animals were housed in individual cages and quarantined for 7 days before use. On day 0, 2% cholesterol and 6% peanut oil diet (Letica, Barcelona, Spain) was started in 30 animals. One week later, vascular injury was induced in the femoral arteries using nitrogen as previously described (Hernández-Presa et al., 2002). After 1 week, the animals were randomized to receive either 10 mg/kg/day of licofelone (LIC, n=10) (provided by Merckle-Germany, Lacer Spain) or 5 mg/kg/day of rofecoxib (ROF, n=10) mixed with chow. A third group did not receive any treatment (NT, n=10). The atherogenic diet was maintained in all cases. Four weeks later, they were sacrificed. Ten rabbits feeding standard chow without intervention were included in the study as healthy controls.

At sacrifice, the animals were anesthetized and the femoral arteries exposed. Then, they were sacrificed with an overdose of pentobarbital (Abbott, Madrid, Spain) and one of the femoral arteries was fixed by perfusion of the descending aorta with 4% buffered formaldehyde at 100 mmHg, removed and kept for 24 h in 4% buffered formaldehyde and afterwards in 70% ethanol until paraffin embedded.

Serum chemistry

Rabbits were bled from a marginal ear vein 24 h post-meal on day 0 and at the end of weeks 2 (randomization) and 6 (sacrifice) of the study. Total cholesterol, chylomicrons, low-density lipoproteins (LDL), high-density lipoproteins (HDL), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and triglycerides (TG) were measured with enzymatic assays (Sigma Diagnostics, Madrid, Spain).
**PGE$_2$ plasma levels**

Ten mL of blood were drawn from ear vein at sacrifice. Plasma was obtained from blood by centrifugation (2,500 rpm, 10 min) and frozen at -80°C. PGE$_2$ levels were measured by a competitive immunoassay (R&D Systems). Total concentration of PGE$_2$ was expressed as pg/mL.

**Histological analysis**

**Morphometric analysis**

The morphometric analysis was performed on Masson-stained femoral artery preparations. Intima and media area were measured and the results were expressed as intima/media ratio.

**Immunohistochemistry**

Paraffin-embedded arteries were cross-sectioned into 4 µm-thick pieces at 5-mm intervals, dewaxed and rehydrated. For macrophage identification, a monoclonal anti-rabbit macrophage antibody (RAM11, DAKO) was applied as described previously (Hernández-Presa et al., 2002). COX-1 and COX-2 were detected with polyclonal goat anti-human antibodies (Santa Cruz Biotechnology). 5-LOX were immunolocalized using polyclonal rabbit anti-human antibody (Cayman). As secondary antibody, a donkey anti-goat peroxidase (The Binding Site, Birmingham, UK) was used for COX-2 and COX-1, a donkey anti-goat IgG biotin labeled (Amersham), a goat anti-mouse IgG biotin labeled (DAKO) for RAM11, and anti-rabbit IgG biotin labeled for 5-LOX. The secondary antibodies were applied for 1 h at 1:200 dilution. Then, ABComplex/HRP (DAKO) was added for a further 30-min period. Then, ABComplex/HRP (DAKO) was added for a further 30-min period. The sections were stained for 10 min at room temperature with 3, 3′- diaminobenzidine tetrahydrochlorure (DAB; DAKO) and were finally counterstained with hematoxylin, and mounted in Pertex (Medite).

In each experiment, negative controls without the primary antibody or using a non-related antibody, were included to check for non-specific staining.
Southwestern histochemistry

The distribution and DNA-binding activity of NF-κB in situ was detected as described previously (Hernández-Presa et al, 2002), using a digoxigenin labeled double stranded DNA probe with a specific consensus sequence which binds to NF-κB (5´-AGTTGAGGGGACTTTCCCAGGC-3´). Preparations without probe were used as negative controls and, while to test the specificity of the technique, a mutant NF-κB probe was used (5´-AGTTGAGGCTCCTTTCCCAGGC-3´).

Image analysis

Preparations were digitalized via an Olympus microscope (BH-2) connected to a CCD video camera as previously described (Hernández-Presa et al., 2002). Image analysis was performed using the Olympus software. For morphometric analysis, intima and media areas were measured. For immunohistochemistry specimens, the percentage of neointima staining positive per mm² was evaluated. In Southwestern preparations, nuclei staining positive per mm² were assessed.

Western blot analysis

The protein levels of COX-1, COX-2 and 5-LOX in femoral arteries were determined by Western blot analysis with specific antibodies (Santa Cruz Biotechnology for COX-1/-2 and Cayman for 5-LOX). 50 µg of whole proteins were separated on a 10-15% SDS-PAGE and transferred to a PVDF membrane (Immobilon P, Millipore). The membrane was blocked in washing solution with 7% nonfat dried milk for 60 min at 37°C and then incubated with 1mg/mL of primary antibody overnight at 4°C and later with a peroxidase-conjugated secondary antibody for 60 min at 37°C. The bands were detected with a chemiluminescent system (ECL, Amersham) and exposed to X-ray film.
RNA extraction and Real-Time Polymerase Chain Reaction

Total RNA was extracted from femoral arteries by Trizol method (Life technologies) and quantified by absorbance at 260 nm in duplicate. 1 µg of RNA was necessary to perform the reverse transcription reaction, for 15 min at 25ºC and 2h at 37ºC, with the High Capacity cDNA Archive Kit (Applied Biosystems). Oligonucleotide primers/probe for rabbit MCP-1 and COX-2 were designed using the Primer Express program and were synthesized by Applied Biosystems. Amplification of Eukaryotic 18S RNA was used in the same reaction of all samples as an internal control. Gene-specific mRNA was subsequently normalized to 18S RNA. Quantitative RT-PCR was performed by 7500 Real Time PCR System and the relative quantification was performed with the Prism 7000 System SDS Software (Applied Biosystems).

Ex vivo studies

Isolation of rabbit neutrophils and LTB4 measurement.

Heparinized peripheral blood was collected from a total of 6 healthy rabbits and neutrophils were isolated by using a lymphocyte separation medium gradient, gelatine sedimentation and hypotonic lysis of erythrocytes as previously described (Lee et al., 2005). Briefly, the sedimentation of the cellular portion was developed by mixing with 2.5% gelatin in phosphate-buffered saline (PBS) and kept over 20 min. at 37ºC. The supernatant of the suspension was harvested and centrifuged at 2500 rpm for 15 min. and then the residual erythrocytes from the pellet obtained were removed by hypotonic lysis and neutrophils population was isolated with a purity of more than 95%.

Neutrophils were resuspended in serum free medium RPMI (25x10^4/300µl) and preincubated 60 min. at 37ºC with licofelone 10μM, rofecoxib 10μM and MK-886, a 5-lipoxygenase inhibitor, at 0.1μM concentration. 10μM calcium ionophore A23187 (10min. at 37ºC) was used to stimulate the LTB4 release in the natural poblation of neutrophils isolated from whole blood. After stimulation cell-free supernatants were collected and centrifuged (1000 rpm, 5min.) and LTB4 concentration was measured by using the comercial enzyme-linked
inmunoassay (EIA) (Cayman, Ann Bor.) according to the manufacturer’s instructions. This kit is highly specific for LTB4. The intra- and inter-assay variability has been determined at multiple points on the standard curve. LTB4 intra-assay variation is between 13% and 18% and inter-assay variation is between 7% and 18%. Total concentration of LTB4 was expressed as pg/mL.

Isolation of rabbit platelets and TXB2 levels measurement.

In order to assess platelet TXB2 levels, the stable metabolite of TXA2, venous blood from healthy rabbits (n=4) was collected in sodium citrate solution 3, 8%. Platelet-rich plasma (PRP) was isolated from whole blood by centrifugation (600 rpm for 30 min) and washed platelet suspension was distributed in samples of 1ml PRP. The PRP was incubated for 60 min at 37°C with different drugs: licofelone, rofecoxib and SC-560, a COX-1 inhibitor for 1 h, at 37°C in agitation. After pretreatment with these drugs, platelet-rich plasma was incubated with 0, 5 U/ml thrombin for 10 min, at 37°C in agitation. Finally, we measured platelets TXB2 production by the EIA method. This kit is highly specific for TXB2 measurement. The intra- and inter-assay variability has been determined at multiple points on the standard curve. TXB2 intra-assay variation is over 18% and inter-assay variation is between 15% and 20%. Total concentration of TXB2 was expressed as pg/mL.

In vitro studies

Cell cultures

Rat VSMC were isolated and cultured as explained previously (Hernández-Presa et al., 1997). Cells were growth-arrested by incubation in serum-free medium for 48 h, and then incubated with the corresponding stimuli. For inhibition studies, licofelone, rofecoxib, SC-560 and MK-866 were added to the culture medium 1 h prior to the stimuli.

RNA extraction and Real-Time Polymerase Chain Reaction

Total RNA extraction of different experiments in VSMC was performed as commented above. Oligonucleotide primers/probe for rat MCP-1 were designed using the Primer Express
program and were synthesized by Applied Biosystems. Amplification of Eukaryotic 18S RNA was used in the same reaction of all samples as an internal control.

**Statistical Analysis**

Statistical analysis was performed with GraphPAD InStat (GraphPAD Software). Lipid values, morphometric analysis, immunohistochemistry, Western blot, Real Time PCR, ELISA and EIA data are presented as mean ±SEM and were analyzed by the U Mann–Whitney test. When multiple comparisons were needed, the Kruskal–Wallis test was used.
RESULTS

Plasma lipid levels

The atherogenic diet increased all lipid parameters, except HDL, which was not altered by any treatment. Total cholesterol and VLDL showed the more pronounced increase. Maximum lipid levels were reached at sacrifice (week 6) and there were not statistical differences among NT, LIC and ROF groups (Table 1).

Licofelone reduces neointimal formation of rabbit atheroma

Licofelone significantly reduced intima/media ratio in injured femoral arteries as compared to non treated animals, (0.2±0.1 vs 0.7±0.2; \(p<0.05\)), while rofecoxib did not modify the neointimal size in the animal model (0.5±0.2 vs 0.7±0.2; \(p=ns\) vs NT) (Figure 1).

Macrophage infiltration was attenuated with licofelone in rabbit atheroma

Licofelone attenuated macrophage infiltration in the neointimal area of the artery in relation to NT group (4±3 % vs 13±5 % of positive staining area; \(p<0.05\)). Rofecoxib did not significantly reduced the macrophage presence (8±4% of positive staining area; \(p=ns\)) in the vascular lesion as compared to non treated animals. As expected, healthy rabbit vessels did not stain for macrophages (Figure 2).

Licofelone diminishes NF-κB activity in vascular lesions of atherosclerotic rabbits

The activation of the nuclear factor NF-κB in the area of vascular lesion of the artery was markedly reduced by LIC as compared to NT group of rabbits (1967±483 vs 3548±324 positive nuclei staining per \(\text{mm}^2\); \(p<0.05\)), while a not significant inhibition was observed in ROF animals (2987±583 nuclei staining per \(\text{mm}^2\); \(p=ns\) vs NT). Control animals did not show NF-κB activation (Figure 3).
Licofelone and rofecoxib reduce MCP-1 expression in vascular lesions and in cultured
VSMC

In rabbit vascular lesions, both licofelone and rofecoxib markedly reduced MCP-1 mRNA expression compared to those values of non treated animals (60±0.8 and 47±1.9 % of inhibition respectively; p<0.01 vs NT) (Figure 4A).

In cultured VSMC, interleukin-1β caused an increase of MCP-1 mRNA. LIC at 10μmol/L and 1μmol/L concentration was able to reduce it (79±0.1 and 63±0.4 % of inhibition respectively; p<0.01 vs stimulus). At the same concentration (10μmol/L and 1μmol/L), ROF inhibited MCP-1 mRNA expression (66±0.3 % of inhibition; p<0.01 and 68±0.4 % of inhibition; p<0.05 respectively) (Figure 4B). In order to confirm the 5-LOX pathway implication in MCP-1 mRNA expression, we performed additional experiments with the 5-LOX inhibitor MK-866 (1μmol/L). This compound inhibited MCP-1 expression (66±0.2 % of inhibition; p<0.01) elicited by IL-1β in VSMC (Figure 4B).

Atheroma 5-LOX, COX-1 and COX-2 expression in LIC and ROF-treated rabbits

The effect of licofelone and rofecoxib on 5-LOX, COX-1 and COX-2 protein expression was measured in the neointimal area of rabbit arteries by western blot (Figure 5) and immunohistochemistry (Figure 6).

Licofelone reduced markedly 5-LOX protein levels by Western blot (0.8±0.12 vs 4.7±2; p<0.01 vs NT and 0.8±0.12 vs 5.5±1.8; p<0.05 vs ROF) and by immunohistochemistry (19±6.4 vs 47±5 % of positive staining/mm²; p<0.05 vs NT) in the neointimal area while, as expected, RFC did not.

As expected, there were no differences between licofelone and rofecoxib on COX-2 and COX-1 expression. Licofelone and rofecoxib showed a trend to inhibit COX-2 protein expression (Figure 5) and mRNA expression (data not shown) although it did not reach to the statistical significance. This tendency was verified by immunohistochemistry, where COX-2 expression in the neointima was significantly inhibited by licofelone (21±5 % vs 39±6 % of
positive staining/mm²; p<0.05 vs NT) and rofecoxib (23±10 % vs 39±6 % of positive staining/mm²; p<0.05 vs NT). COX-1 expression showed a significant reduction of this enzyme in the neointima by licofelone (28±5 % vs 37±4 % of positive staining/mm²; p<0.05).

**PGE₂ plasma concentration was decreased by both licofelone and rofecoxib in rabbits.**

The concentration of PGE₂ in rabbit plasma was increased in NT as compared with healthy animals (5,300±66 pg/mL; p<0.001 vs healthy). These levels returned to the normal values of healthy animals by licofelone (1,057.5±191.3 pg/mL, p<0.01 vs NT) and rofecoxib (1,603.1±344.6 pg/mL, p<0.01 vs NT) treatment (Figure 6A).

**Licofelone inhibits LTB₄ production in rabbit neutrophils.**

To examine the involvement of licofelone on 5-LOX activity we assessed the effect of licofelone and rofecoxib on LTB₄ generation ex vivo in rabbit neutrophils. After the optimization of assay conditions for the maximal LTB₄ release by the neutrophils isolated from peripheral blood of healthy rabbits, the stimulation was performed with A23187 at 20µM concentration for 10min. at 37ºC. Neutrophil preincubation with licofelone at 10µM concentration inhibited LTB₄ release (9.6±7 pg/ml; p<0.01 vs stimulus; p<0.001 vs ROF) stimulated by calcium ionophore A23187 20µM. Licofelone diminished the levels of the proinflamatory leukotrienes at values similar to basal concentration. The same effect was seen with the 5-lipoxygenase inhibitor MK-886 10µM (25.4±44 pg/ml; p<0.01 vs stimulus). However, none of the rofecoxib doses tested (0.1µM, 1µM, 10µM) has an inhibitory effect on LTB₄ production by neutrophils after the stimulation with A23187 (200.5±19.1pg/ml; p=ns vs NT) (Figure 6B).

**Licofelone prevents TXB₂ production in rabbit platelets.**

In order to assess whether licofelone treatment affects platelet COX-1 expression and TXA₂ production in rabbits, we isolated platelet-rich plasma (PRP) from whole blood of healthy
rabbits (n=4) and we measured platelet TXB₂, as an index of TXA₂ production in vivo. Platelets were pretreated with licofelone, rofecoxib and SC-560 for 1 h prior to stimulation with thrombin for 10 min. Thrombin (0.5U/mL) significantly increased platelet TXB₂ production (669±85 vs 41±13 pg/mL; p<0.05 vs control). Licofelone at 10µmol concentration and SC-560 10µmol inhibited the COX-1 pathway, reducing noticeably platelet TXB₂ production elicited by thrombin (162±50 pg/mL and 224±77 pg/mL; p<0.01 in both cases vs stimulus). Rofecoxib failed to modify platelet TXB₂ production induced by thrombin (468±113 pg/mL, p=ns vs stimulus) (Figure 6C).
DISCUSSION

Inflammation plays an essential role in the development and rupture of atherosclerotic plaques (Willerson and Ridker, 2004). Cyclooxygenase (COX) is the rate-limiting enzyme in the biosynthesis of prostanoids, which exert a variety of actions on the vascular wall. COX-2 isoform is a key mediator of inflammation, upregulated in activated monocyte/macrophages, suggesting that COX-2 inhibition might reduce atherogenesis through its anti-inflammatory effects (Linton et al., 2004). However, in the last years there has been great interest in the possible antiatherogenic effects of COX-2 inhibitors. Clinical studies have raised concern about the safety of these drugs because not only a reduction, but also a lack of effect or even an increase in the incidence of cardiovascular events was observed (Bombardier et al., 2000; Silverstein et al., 2000; Altman et al., 2002; Chevenard et al., 2003; Bresalier et al., 2005; Solomon et al., 2005). It was assumed that COX-2 inhibitors would cause an imbalance between thromboxane A₂ and prostacyclines, favoring platelet aggregation, vasoconstriction, and thrombosis. However, PGs are not the only metabolites of arachidonic acid. The enzyme 5-LOX, localized in macrophages and other inflammatory cells, converts arachidonic acid into proinflammatory leukotrienes (LTs) (Steinhilber, 1999).

In the last years, several new 5-LOX and COX-2 inhibitors have been developed (Martel-Pelletier et al., 2003) in a search for an anti-inflammatory compound with higher gastrointestinal safety than the classical non-steroidal anti-inflammatory drugs and without the cardiovascular risk associated to COX-2 inhibitors. Licofelone is one of these potent anti-inflammatory drugs.

In our experimental model, licofelone was able to reduce intima/media ratio in injured rabbit arteries whereas the selective COX-2 inhibitor rofecoxib did not show a significant reduction of the neointimal size in this animal model. Therefore, Licofelone could be able to interfere with the process of neointimal growth linked to vascular injury. Indeed, lipoxygenase products of arachidonic acid, especially HETEs, have been shown to play an important role in neointimal formation after vascular injury (Fujita et al., 1999).
Neointimal macrophage infiltration in the atherosclerotic lesions in rabbits was attenuated by licofelone, but not by rofecoxib. We have also demonstrated that the action of licofelone on reducing the inflammatory cells in the atherosclerotic process could also be mediated through its inhibition of MCP-1, which participates in the atherosclerotic process by the recruitment of monocytes into the arterial wall. We showed that licofelone inhibits MCP-1 expression in cultured VSMC in an inflammatory state probably via COX-2 inhibition, as it has been reported that COX-2 inhibition decreases MCP-1 expression (Wang et al., 2005), as well as by 5-LOX blocking, since the inhibitor of this enzyme MK-866 also reduced MCP-1 expression in the presence of proinflammatory cytokines, although other factors must be interfering in this process. Therefore, it is tempting to suggest that the enhanced inhibitory effect of licofelone on MCP-1 gene expression could be mediated not only via COX-2, but also through 5-LOX. In this regard, LTB4 derived from 5-LOX activity, binds to its high-affinity receptor BLT1, promoting monocyte adhesion by increasing MCP-1 expression (Friedich et al, 2003; Huang et al, 2004).

The nuclear factor NF-kB is a key mediator of inflammation that regulates many genes involved in cell recruitment (Barnes et al., 1997; Martin-Ventura et al., 2004). Since licofelone inhibited NF-kB in vascular lesions and the modulation of NF-κB regulates the transcription of a number of genes as COX-2 and 5-LOX, among others, we assessed the effect of licofelone and rofecoxib on protein expression of COX-2, COX-1 and 5-LOX in rabbit vascular atheroma. Licofelone and rofecoxib showed a similar inhibitory effect on COX-1 and COX-2 protein expression. Conversely, 5-LOX expression was only altered by licofelone, which exerted a profound lessening of the levels of this enzyme compared to non-treated animals and rofecoxib group. The inhibitory effect of licofelone on 5-LOX expression in vascular lesions was associated to the lessening of the activated NF-kB observed in vascular atheroma of licofelone-treated rabbits. On the other hand, licofelone could have an inhibitory effect on NF-kB through the inhibition of 5-LOX by a feedback mechanism. In this regard, it has been reported that arachidonic acid-derived metabolites as LTB4, acting through its G-protein-coupled receptor in
a feed back mechanism, contribute to the activation of NF-κB in response to TNF-α and IL-1β (Anthonsen, 2001).

Therefore, although there is an important contribution of COX-2 and 5-LOX protein inhibition in the anti-inflammatory action of licofelone, we also characterized the impact of this drug on the generation of the eicosanoids derived from the activity of COX and 5-LOX enzymes under proinflammatory conditions, as a measure of the activity of these enzymes.

The proinflammatory metabolite PGE2, a product of COX-2 activity, was reduced in the same way by both licofelone and rofecoxib in rabbit plasma, which is in agreement with the lessening of COX-2 expression by both drugs in rabbit atheroma. The most important eicosanoids resulting from 5-LOX activity is LTB4, which exhibits strong proinflammatory activity in cardiovascular tissues and enhance vascular permeability and endothelial dysfunction. LTB4, a potent leukocyte chemoattractant, is also involved in cytokine synthesis, regulation of lymphocyte proliferation, and macrophage cytotoxic and natural killer cell activities (Vila, 2004). LTB4 is also an important regulator of neutrophil function and causes chemotaxis, degranulation, phagocytosis and superoxide generation in neutrophils (Tager et al., 2003). In this sense, we have studied LTB4 generation by neutrophils isolated from whole blood of rabbits as a measure of 5-LOX activity. The results obtained in our study are of potential interest, since licofelone at 10µM concentration almost abolished the production of the proinflammatory LTB4 after stimulation with calcium ionophore. This fact confirms that licofelone is able to interfere with the enzymatic activity of 5-lipoxygenase under proinflammatory conditions.

The strong inhibitory effect of the 5-LOX inhibitor MK-886 at 10µM concentration was similar to that produced by licofelone. Actually, it has been reported that the effects of MK-886 are associated with a profound inhibition of ex-vivo LTB4 synthesis in blood and a significant reduction of neutrophil aggregation in whole blood (Lee et al., 2005). The significant effects of MK-886 are mainly due to inhibition of neutrophil function and this suggests an important modulatory role for leukotrienes, and obviously for 5-LOX, in the pathology of inflammatory disease. In contrast, when neutrophils were incubated in presence of rofecoxib, the levels LTB4
remained elevated compared with the stimuli. Consequently, the use of a selective inhibitor of COX-2 similar to rofecoxib, in vascular inflammatory states would lead to a decrease in antithrombotic prostacyclin synthetized by arachidonate flux through COX-2, and more arachidonic acid would be available for leukotriene synthesis. Indeed, it has been published that the inhibition of COX and PGE2 synthesis could be responsible for the increased levels of LTB4 production by human osteoarthritic joint tissues (Martell-Pelletier et al., 2004). As a result, the overproduction of LTB4 would cause inflammatory responses that may result in the migration and adhesion and of inflammatory cells by increasing MCP-1 expression (Friedich et al., 2003; Huang et al., 2004) in agreement with the results.

In the present study have also confirm the protective antithrombotic effect of licofelone, since the concentration of TXB2, the degradation product of the prothrombotic TXA2, was diminished in platelets isolated from peripheral blood of rabbits. This effect was mainly due to COX-1 inhibitory activity of this drug, since the COX-1 specific inhibitor SC-560 had similar results. On the other hand, the selective inhibition of COX-2 by rofecoxib was not able to reduce TXB2, and consequently its levels remained elevated, disrupting the physiological balance between thromboxane and prostacyclin and, consequently, augmenting thrombogenesis, and the risk of cardiovascular complications (Linton et al., 2004).

In a rabbit model of atherosclerosis, as well as in cultured cells, we have demonstrated the protective effect of licofelone on the atherosclerotic process in comparison with rofecoxib as shown by others (Moreau et al., 2005). We have also confirmed the anti-inflammatory and antithrombotic properties of licofelone in neutrophils and platelets isolated from whole blood.

On the whole, our findings disclose that licofelone, in addition to exert a beneficial anti-inflammatory effect on the atherosclerotic lesions, it could also avoid the cardiovascular risk associated to selective COX-2 inhibitors due to its inhibitory effect on prothrombotic TXA2. Therefore, licofelone could have a safer cardiovascular profile than COX-2 inhibitors in inflammatory diseases although clinical trials are needed to confirm this issue.
REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

**Figure 1. Morphometric analysis of femoral arteries.** The morphometric analysis was performed on Masson stained arterial preparations. Intima/media ratio was measured. The neointimal formation was reduced by licofelone while rofecoxib failed to diminish the neointimal size. * p<0.05 vs NT.

**Figure 2. Representative examples of immunohistochemistry of RAM11 in the lesions.** An increase of positive staining in the neointimal area in NT group and a significant reduction in licofelone group is shown. Macrophage infiltration in vascular lesions was attenuated by licofelone whereas rofecoxib was unable of preventing the macrophages presence in rabbit atheroma. * p<0.05 vs NT.

**Figure 3. Effect of licofelone and rofecoxib on the activation of NF-κB in femoral arteries.** Representative examples of Southwestern histochemistry of NF-κB activity in the lesions, showing a great number of nuclei staining positive in NT animals, which was reduced in rabbits treated with licofelone and absent in healthy animals. *p<0.05 vs NT.

**Figure 4. Effect of licofelone and rofecoxib on MCP-1 mRNA expression in femoral vascular lesions and in VSMC.** (A) Analysis by Real Time PCR of the MCP-1 mRNA expression of femoral artery. Both licofelone and rofecoxib reduced MCP-1 gene expression in rabbit vascular atheroma. * p<0.05 vs healthy, †† p<0.01 vs NT. (B) Analysis of the MCP-1 mRNA expression by Real Time PCR in rat VSMC cultured. Licofelone and rofecoxib, at 10µmol/L, and MK-886, 1µmol/L, inhibited notably MCP-1 expression. **p<0.01 vs control; † p<0.05 vs stimulus, †† p<0.01 vs stimulus.
Figure 5. Atheroma 5-LOX, COX-1 and COX-2 protein expression in licofelone and rofecoxib-treated rabbits. Western blot analysis of 5-LOX, COX-1, COX-1 and protein expression in femoral arteries. 5-lipoxygenase protein expression was almost abolished by licofelone compared with NT and ROF groups. COX-1 expression, as expected by its constitutive nature in almost all tissues, was not altered by any of the treatments administered to the animals. COX-2 enzyme showed a trend to inhibition by both licofelone and rofecoxib in relation to non treated animals although it did not reach statistical significance ** p<0.01 vs NT; † p<0.05 vs ROF.

Figure 6. 5-LOX, COX-1 and COX-2 protein expression in vascular lesions by in situ immunohistochemistry.
Representative examples of immunohistochemistry of 5-LOX, COX-1 and COX-2 in vascular lesions. Both licofelone and rofecoxib were able to decrease COX-2 expression in a significant manner. 5-lipoxygenase protein expression was significantly inhibited by licofelone compared with NT group and as expected, rofecoxib did not. COX-1 expression was reduced by licofelone. † p<0.05 vs NT.

Figure 7. Effect of Licofelone and rofecoxib on COX-2, COX-1 and 5-LOX activity.
(A) PGE₂ plasma levels as a measure of COX-2 activity in atherosclerotic rabbits was reduced by both licofelone and rofecoxib . *** p<0.001 vs healthy, †† p<0.01 vs NT. (B) LTB₄ production measurement in rabbit neutrophils isolated from whole blood of healthy rabbits by EIA method. LTB₄ generation by neutrophils stimulated with the ionophore A23187 was significantly inhibited by licofelone and the 5-LOX inhibitor MK-886. However, LTB₄ concentration in rofecoxib group was similar to that of non treated animals. ** p<0.01 vs stimulus; ††† p<0.001 vs rofecoxib. Ionophore A23187 40µmol/L; LIC=10 µmol/L licofelone; ROF=10 µmol/L rofecoxib and MK=MK-886 1µmol/L. (C) Measurement of COX-1 mediated TXB₂ production in platelet-rich plasma (PRP) from whole blood of healthy rabbits by EIA. Licofelone and the COX-1 inhibitor SC-560 prevented TXB₂ production. * p<0.05 vs control;
†† p<0.01 vs stimulus. Thrombin 0.5U/mL; LIC=10μmol/L licofelone; ROF=10μmol/L rofecoxib; SC=0.1μmol/L SC-560 and MK=10μmol/L MK-866.
Table 1. Plasma lipid parameters (mg/dL).

Data are shown as mean ± SEM. NT, non-treated animals; LIC, Licofelone-treated animals; ROF, rofecoxib-treated animals; TC, total cholesterol; VLDL, very low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; TG, triglycerides; * p<0.05 vs 0 week; † p<0.05 vs healthy.

<table>
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<th>2 weeks</th>
<th>6 weeks</th>
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<tr>
<td></td>
<td></td>
<td>Healthy</td>
<td>NT</td>
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<tr>
<td>TC</td>
<td>54 ± 5</td>
<td>1220 ± 114;</td>
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<td>* †</td>
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<tr>
<td>IDL</td>
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<tr>
<td>LDL</td>
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<td>TG (VLDL)</td>
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Figure 1
Figure 2
Figure 3
Figure 4

A

B

MCP-1 mRNA

( Arbitrary units )

HEALTHY  NT  LIC  ROF

MCP-1 mRNA

( n-Fold vs control )

Control  IL-1β  LIC (10 μM)  LIG (1 μM)  ROF (20 μM)  ROF (1 μM)  ME (1 μM)

IL-1β
**Figure 5**

![Graphical representation of Figure 5 showing protein expression levels for 5-LOX, COX-1, COX-2, and α-tubulin with bar graphs for different conditions: HEALTHY, NT, LIC, ROF.](image-url)
Figure 7

A

![Bar chart showing PC2 levels in different conditions: Healthy, NT, LIC, ROF.](image)

B

![Bar chart showing LT4 levels in different conditions: Basal, LIC, ROF, MK.](image)

C

![Bar chart showing TXB2 levels in different conditions: Basal, LIC, ROF, SC.](image)