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Parecoxib Increases Blood Pressure Through Inhibition of Cyclooxygenase-2 Messenger RNA in an Experimental Model

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ABSTRACT

Background: Cyclooxygenase-2 selective inhibitors have been developed to alleviate pain and inflammation; however, the use of a selective cyclooxygenase-2 inhibitor is associated with mild edema, hypertension, and cardiovascular risk. **Aim:** To evaluate, in an experimental model in normotensive rats, the effect of treatment with parecoxib in comparison with diclofenac and aspirin and L-NAME, a non-selective nitric oxide synthetase, on mean arterial blood pressure, and cyclooxygenase-1 and -2 messenger RNA and protein expression in aortic tissue. **Methods:** Rats were treated for seven days with parecoxib (10 mg/kg/day), diclofenac (3.2 mg/kg/day), aspirin (10 mg/kg/day), or L-NAME (10 mg/kg/day). Mean arterial blood pressure was evaluated in rat tail; cyclooxygenase-1 and -2 were evaluated by reverse transcription-polymerase chain reaction and Western blot analysis in aortic tissue. **Results:** Parecoxib and L-NAME, but not aspirin and diclofenac, increased mean arterial blood pressure by about 50% ($p < 0.05$) without changes in cardiac frequency. Messenger RNA cyclooxygenase-1 expression in aortic tissue was not modified with any drug ($p < 0.05$). L-NAME and parecoxib treatment decreased messenger RNA cyclooxygenase-2 and cyclooxygenase-2 ($p < 0.05$). While cyclooxygenase-1 protein decreased with the three drugs tested but not with L-NAME ($p < 0.05$), the cyclooxygenase-2 protein decreased only with aspirin and parecoxib ($p < 0.05$). **Conclusion:** Parecoxib increases the blood pressure of normotensive rats by the suppression of *COX-2* gene expression, which apparently induced cardiovascular control. (REV INVES CLIN. 2015;67:250-7)

Key words: Parecoxib. Hypertension. Cyclooxygenase-2. Prostaglandin I_2. Thromboxane A_2. mRNA inhibition.

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INTRODUCTION

Cyclooxygenase-2 (COX-2) selective inhibitors have been developed to alleviate pain and inflammation, based on the finding that cyclooxygenase-1 (COX-1) is involved in the physiology of the gastrointestinal mucosa[1,2] and is different from COX-2, which is induced by inflammation[3]. Therefore, selective inhibition of COX-2 could dissociate anti-inflammatory activity from the gastrointestinal side effects of the non-steroidal anti-inflammatory drugs (NSAID, non-selective COX)[4,5].

The selective inhibition induced by COX-2 may cause gastrointestinal side effects that are of lesser importance compared with the cardiovascular side effects in relation to the physiological balance between the prothrombotic and vasoconstrictor actions of COX-1 and COX-2. The COX-1 is derived from thromboxane A2 (TXA2) in platelets, and the anti-aggregation and vasodilation actions of COX-2 derive from prostaglandin I2 (prostacyclin, PGI2). Inhibition of COX-2 in endothelium in favor of platelet aggregation may implicate cardiovascular risk[6,7]. The original hypothesis was that COX-2 inhibition only affects proinflammatory prostaglandins; however, this was soon questioned and the discovery that COX-2 inhibition in humans suppressed the systemic biosynthesis of prostacyclin represented a breakthrough in the risk-benefit assessment of coxibs[8]. Prostaglandin I2 is a potent vasodilator and platelet inhibitor produced in blood vessels by the enzymatic activity of COX-1 and COX-2 and prostacyclin synthase. Thromboxane A2 has been shown *in vitro* and *in vivo* to modulate vasoconstrictor and platelet aggregator activities, and this COX-derived prostanoid is produced mainly by platelets activated via COX-1 during hemostasis[9].

The use of a selective COX-2 in healthy individuals is associated with mild edema and hypertension due to modest sodium retention in the first days of therapy[10]. Thus, studies have been conducted to analyze the risks arising from these drugs, including Vioxx Gastrointestinal Outcomes Research (VIGOR; rofecoxib vs. naproxen)[11], the Celecoxib Long-term Arthritis Safety Study (CLASS; celecoxib vs. diclofenac and ibuprofen)[12], the Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL; etoricoxib vs. diclofenac in a pooled analysis), and the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET; lumiracoxib vs. ibuprofen and naproxen)[4]. The studies showed that COX-2 inhibitors celecoxib and rofecoxib increase cardiovascular risks compared with placebo, although the studies do not clearly define the mechanisms for cardiovascular risk development. Parecoxib is one of the drugs in this group and is the first injectable COX-2 selective inhibitor indicated for the treatment of acute postoperative pain; it is an inactive prodrug that undergoes rapid amide hydrolysis *in vivo* into the pharmacologically metabolite-active valdecoxib[13]. This drug has had widespread use because it offers an advantage over the other coxibs and has good analgesic effect in postoperative pain. As such, it comprises a good therapeutic option in acute dental pain and in orthopedic and gynecological pain; however, many authors suggest that valdecoxib and parecoxib are both efficacious and well tolerated[14]. At present, three coxibs (celecoxib, etoricoxib, and parecoxib) are authorized and marketed in several countries, with parecoxib preferably for hospital use[15].

The adverse cardiovascular events observed in experimental models and in patients under selective COX-2 inhibition therapy could be explained by either a TXA2/PGI2 imbalance or changes in the expression and/or activity of the COX isoforms. Therefore, in this study we developed an experimental model to evaluate the effect of the selective COX-2 inhibitor parecoxib in comparison with the non-selective COX inhibitors diclofenac and aspirin, as well as water (negative control) and L-NAME, a non-selective nitric oxide synthetase (as positive control of hypertension) on mean arterial blood pressure (MABP), and COX-1 and COX-2 messenger RNA (mRNA) and protein expression in the aortae of normotensive rats.

MATERIALS AND METHODS

Animals

Male Wistar Kyoto rats weighing 200-250 g each were housed in an environmentally controlled room with a 12-hour/12-hour light/dark cycle; they were given standard rodent chow and tap water *ad libitum*. All experimental procedures described here were approved by the local Animal Care Committee of the Autonomous University of San Luis Potosi and followed the Declaration of Helsinki principles.

Treatments

The rats were acclimated to handling by humans prior to randomization, and then were divided into the following four groups of six rats each: (i) the control group received untreated drinking water; (ii) parecoxib group (Dynastat®, Pfizer Co., México), 10 mg/kg/day; (iii) diclofenac group (Artrenac, Merck Co., México), 3.2 mg/kg/day; (iv) aspirin group (Pisa Laboratorios, México), 10 mg/kg day; and (v) L-NAME group (L-NAME, Sigma-Aldrich Co.), 10 mg/kg day. All experimental drugs were administered in the animals' drinking water, and the treatments were given for up to seven days.

Mean arterial blood pressure measurements

Mean arterial blood pressure was measured daily by using the tail-cuff method with an LE 5002 Storage Pressure Meter (Letica Scientific Instruments, USA). Previous to treatment, the rats underwent a one-week adaptation period to avoid stress during measurements. Rats were placed in a temperature-controlled restriction chamber at 28°C during 10 minutes. The cuff was inflated automatically by means of a retroserver, and the pressures measured fell within the range of 30-300 mmHg. From each rat, heart rate, systolic blood pressure (SBP), and diastolic blood pressure (DBP) values were obtained. The MABP was calculated by the instrument that integrates the difference between SBP and DBP in terms of time, which is calculated by means of the following equation: MABP = (DBP + SBP)/3. This procedure was repeated five times, every two minutes, during the 10-minute restriction time. Results are expressed in mean mmHg ± standard error of the mean (SEM) (n = 6).

Total RNA isolation reverse transcription

At the end of the treatment period, the animals were euthanized by overexposure to the anesthetic (ether vapor). The thoracic aorta was immediately excised and placed on a phosphate-buffered saline (PBS)-precooled plate. The aortic tissue was dissected from the adherent fat and connective tissues and was maintained on ice, then frozen in liquid nitrogen, and maintained at −80°C for subsequent analysis. Fifty milligrams of thoracic aorta was dissected and immediately homogenized in 1 ml of Trizol™. Total RNA was isolated using 200 µl of chloroform, and the samples were centrifuged at 12,000 rpm for 15 minutes at 4ºC. The supernatant was collected, the RNA was precipitated with 500 µl of isopropanol for 10 minutes at −20ºC, and the integrity of the nucleic acid was evaluated by denaturing electrophoresis.

Reverse transcription of 5 µg of total RNA was performed using Oligo (dT) 12-18 primer (Invitrogen™) and the Moloney murine leukemia virus (M-MLV) reverse transcriptase enzyme (Invitrogen™) during one hour at 37ºC. Subsequently, the complementary DNA (cDNA) of COX-1 and COX-2 was amplified by a specific reverse transcription-polymerase chain reaction (RT-PCR) using the following primers (Life Technologies), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the in-house control gene: COX-1 forward, 5'TAA-GTA-CCA-GGT-GCT-GGA-TGG; COX-1 reverse, 5'GGT-TTC-CCC-TCT-AAG-GAT-GAG-G; COX-2 forward, 5'TAC-AAG-CAG-TGG-CAA-AGG-C; COX-2 reverse, 5'CAG-TAT-TGA-GGA-GAA-CAG-ATG-GG; GAPDH forward, 5'AAC-ACA-GTC-CAT-GCC-ATC-AC, and GAPDH reverse, 5'TTC-ACC-ACC-CTG-TTG-CTG-TA. Thirty cycles of amplification were performed, consisting of denaturing at 94ºC for 60 seconds, annealing at 64ºC for 60 seconds, and extension at 72 ºC for 60 seconds. The PCR amplification products (264 pb, 303 pb, and 360 pb for COX-1, COX-2, and GAPDH, respectively) were analyzed by the optical density of the bands. Results were expressed in arbitrary density units (mean ± SEM) vs. the density of GAPDH.

Protein extraction and Western blot

Homogenates were prepared from 100 mg of tissue as follows: frozen rat thoracic aortae were suspended in 1 ml of cold Tris-hydrochloride buffer (100 mM Tris, pH 7.4) containing a protease inhibitor cocktail (9.9×10^{-3} mM PMSF, 0.09 mM TLCK, 2.07×10^{-3} mM, and 1.25×10^{-3} mM IAA). The tissues were homogenized at 10,000 rpm for two minutes and immediately centrifuged at 1,000 rpm for 10 minutes at 4 ºC in a refrigerated centrifuge (Sorvall Biofuge, Fresco). The pellet was discarded and the supernatant was stored in aliquots at −80 ºC. Total protein concentration was determined by the Bradford micromethod assay (Biorad). Immunoblotting was performed according to a standard protocol. Twenty micrograms per sample of total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were electrotransferred onto

polyvinylidene difluoride (PVDF) membranes in a Trans-Blot Cell (BioRad Labs, Hercules, CA, USA). The membranes were blocked with fresh TBS-T buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.5% Tween 20) containing 5% fat-free milk for one hour at room temperature. The membranes were washed in TBS-T buffer and incubated overnight at 4ºC with the primary antibody against COX-1 (1:200), COX-2 (1:200), and β-actin (1:1,000) (Santa Cruz Laboratories). After three washes, the membranes were incubated for two hours with the horseradish peroxidase (HRP)-secondary antibody diluted at 1:1,000, and a chemiluminescent substrate was added (Luminol, Santa Cruz Laboratories). The bands were quantified by densitometry, and the amount of each product was normalized with respect to the amount of β-actin (load control in Western blot).

Statistical analysis

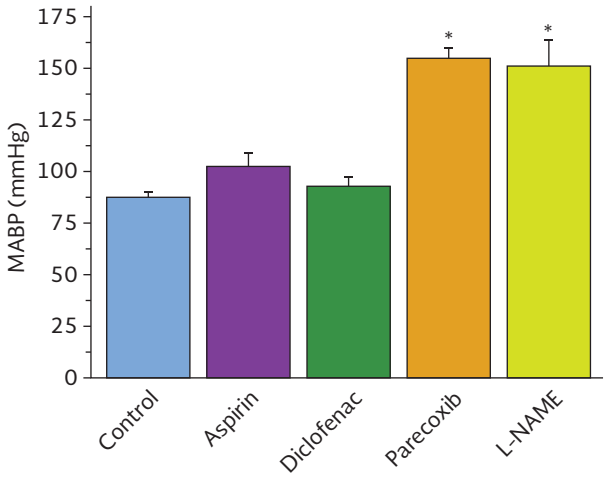
Data are expressed as mean ± SEM and were analyzed with the Dunnett test for analysis of variance (ANOVA) for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Daily oral intake of parecoxib and L-NAME, but not of aspirin and diclofenac, resulted in a progressive increase in BP in normal rats from day 3 of treatment. By day 7 of treatment, MABP was 96 ± 14 mmHg in the control group, 102 ± 7 mmHg in the aspirin group, 97 ± 5 mmHg in the diclofenac group, 150 ± 17 mmHg in the parecoxib group ($p < 0.05$ vs. control), and 155 ± 13 mmHg in the L-NAME group ($p < 0.05$ vs. control). Parecoxib and L-NAME treatments increased MABP by 50% compared with the control group (Fig. 1). The heart rate was not modified throughout the observation period: 409 ± 8 beats/minute in control vs. 380 ± 24 beats/minute in the L-NAME group and 439 ± 16 beats/minute in the parecoxib group, while there were no differences in the aspirin and diclofenac groups vs. the control group (data not shown).

Expression of COX-1 mRNA in aortic tissue was unchanged by any of the treatments (Fig. 2 A). However, *COX-2* gene expression was completely suppressed by parecoxib and L-NAME decreased COX-2 mRNA levels by about 50%. The non-selective COX inhibitors aspirin and diclofenac did not modify COX-2 mRNA expression (Fig. 2 B).

Figure 1. Mean arterial blood pressure (mmHg) of rats (n = 6 per group) after seven days of treatment with the selective cyclooxygenase-2 inhibitor parecoxib compared with the non-selective cyclooxygenase inhibitors diclofenac and aspirin or the non-selective nitric oxide synthetase L-NAME (positive control for hypertension) or water (negative control for hypertension). Bars represent mean ± standard error of the mean (SEM). *Significant differences vs. control group ($p < 0.05$). MABP: mean arterial blood pressure.

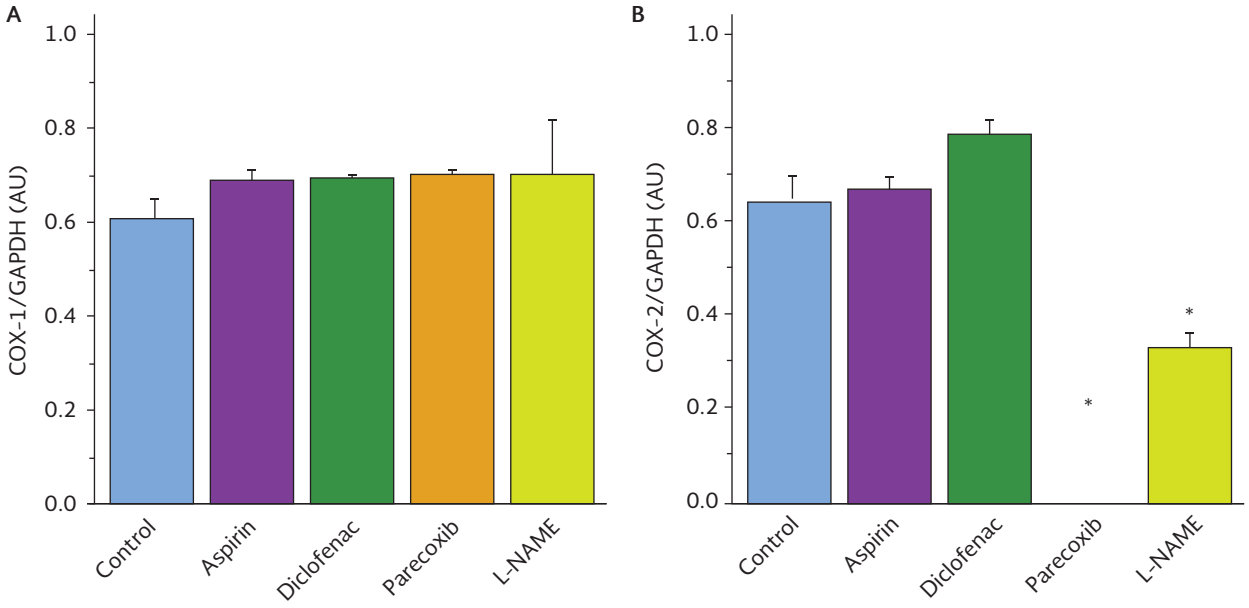


In contrast to the mRNA results, COX-1 protein in aortic tissue was significantly reduced by all COX inhibitors, that is, by parecoxib 68%, diclofenac 40%, and aspirin 72% ($p < 0.05$ vs. control), but no effect was observed with L-NAME (Fig. 3 A). With regard to COX-2, protein expression was reduced by 50% with aspirin and 22% with parecoxib ($p < 0.05$ vs. control), although no changes were observed with the diclofenac and L-NAME treatments (Fig. 3 B). Protein expression of COX-1 was significantly reduced (50%) with aspirin, diclofenac, and parecoxib compared with the control group ($p < 0.05$) (Fig. 3 A).

DISCUSSION

This experimental model was designed to compare the effect of mid-term exposure to selective and non-selective COX inhibitors on BP, and gene/protein expression of COX-1 and COX-2 mRNA in aortic tissue of normotensive rats. Parecoxib increased MABP by suppressing COX-2 at the protein and mRNA levels (Fig. 1). Our study shows that parecoxib is associated with an important increase of MABP, contrary to the study by Chan, et al. in 2009[16], in which the authors reported

Figure 2. A: messenger RNA (mRNA) expression of cyclooxygenase-1 determined by reverse transcriptase polymerase chain reaction in rat aortic tissue on day 7 of treatments. **B:** messenger RNA (mRNA) expression of cyclooxygenase-2 determined by RT-PCR in rat aortic tissue on day 7 of treatments. Bars represent the mean ± standard error of the mean (SEM) (n = 6).
*Significant differences vs. control ($p < 0.05$).
COX: cyclooxygenase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; AU: arbitrary optical density units.



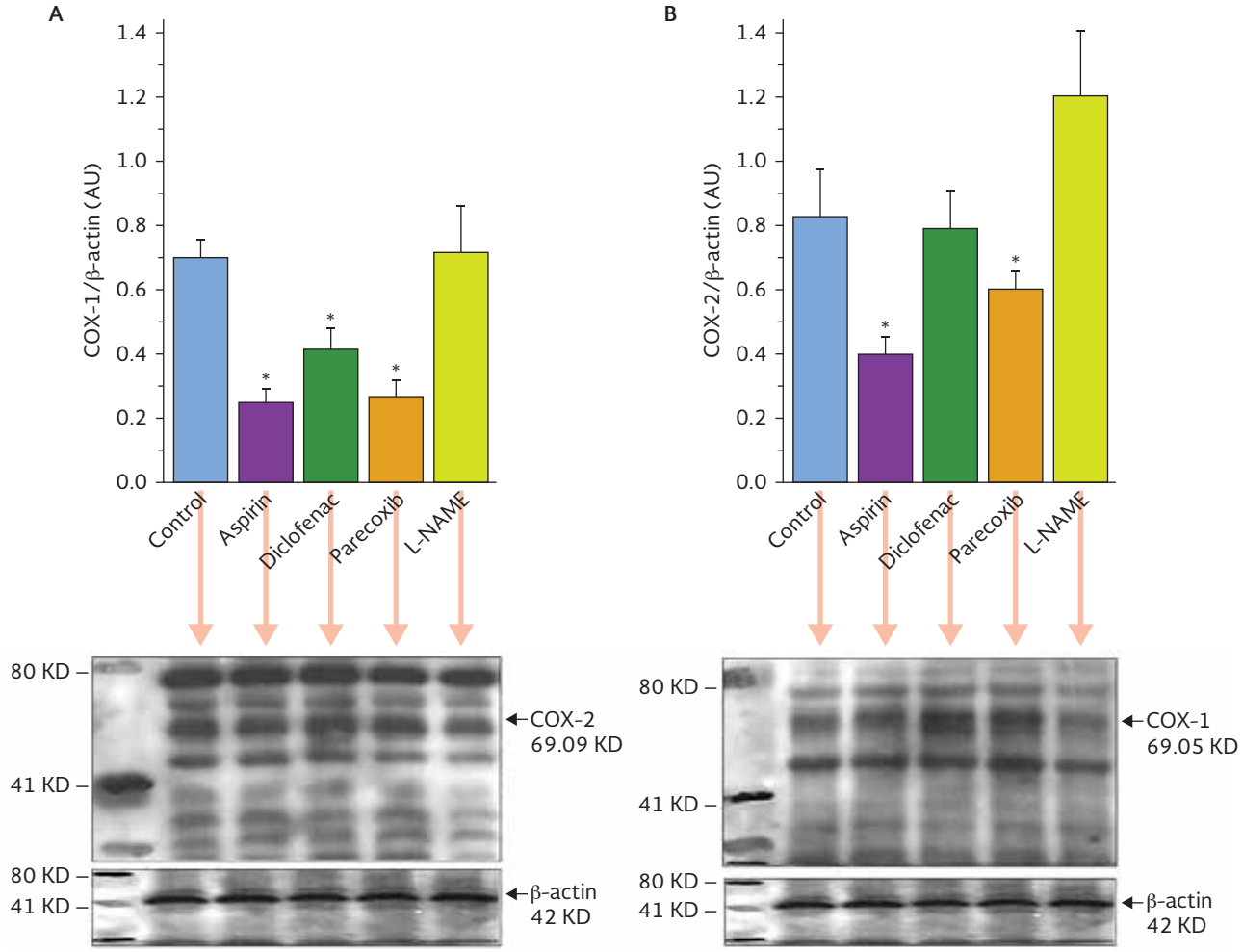
that rofecoxib and etoricoxib were related with hypertension but coxibs had little effect on blood pressure.

Thus, the final effect in MABP appears to be related with PGI2 and TXA2. Rudic, et al. in 2005[17] documented that suppression of COX-2-derived PGI2 or deletion of the PGI2 receptor (IP) profoundly influences the morphological response of the vasculature to hemodynamic stress. Mechanism-based vascular remodeling may interact with a predisposition to hypertension and atherosclerosis, contributing to the gradual transformation of cardiovascular risk during extended periods of treatment with selective COX-2 inhibitors. The COX-2 inhibitors suppress PGI2 biosynthesis without concomitant inhibition of TXA2, which derives predominantly from platelet COX-1.

Indeed, the seven-day treatment with the non-selective COX inhibitors diclofenac or aspirin did not modify MABP. The effect observed may be explained by the selective inhibition of these drugs. Parecoxib produced an increase of nearly 50% in MABP, similar to that observed with the non-selective nitric oxide (NO) synthetase inhibitor L-NAME. It is known that the endothelium regulates vascular tone through the production of NO, and also by prostacyclin and hyperpolarizing factors, thus strengthening the conclusions of studies in humans or in obese Zucker rats, which have reported endothelium dysfunction in several vascular beds associated with reduced NO bioavailability and hypertension[18]. The absence of NO is also associated with reduced cardiac output, cardiac hypertrophy, large areas of fibrosis, and myocardial necrosis, changes in myocardial contractility, and cardiomyocyte and vascular smooth-muscle remodeling[19].

It appears that parecoxib may only be related with inhibition of COX-2, but not of COX-1 (Fig. 2 B and 2 A). COX-2 is an enzyme that may cause metabolic imbalance, resulting in an overproduction of harmful by-products that may damage the arterial wall and induce arterial blood clotting, increasing the risk for thromboembolic events[4]. When COX-2 is inhibited, less PGI2 is synthesized from arachidonic acid and more leukotriene B4 and TXA2 are produced. The PGI2 is both vasodilator and anti-aggregator, while TXA2 is vasoconstrictor and pro-aggregator, and this tip of the balance allows TXA2 to function unopposed, leading to an increased risk for adverse cardiovascular events[20]. These changes may explain the increase in

Figure 3. A: expression of cyclooxygenase-1 in rat aortic cells. Cyclooxygenase-1 expression was determined by Western blot of protein amounts and semi-quantitated by densitometry. Results are the average of six separate experiments expressed as the cyclooxygenase-1/beta actin ratio. **B:** expression of cyclooxygenase-2 in rat aortic cells. Cyclooxygenase-2 expression was determined by Western blot of protein amounts and semi-quantitated by densitometry. Results are the average of six separate experiments expressed as the cyclooxygenase-2/beta actin ratio. Bars represent the mean ± standard error of the mean (SEM) (n = 6). *Significant differences vs. control ($p < 0.05$).
COX: cyclooxygenase; AU: arbitrary optical density units.



heart rate, MABP, and COX-2 mRNA in the parecoxib and L-NAME groups in our study. The cardiovascular safety of coxibs is an important public health issue, considering the large number of predominantly elderly patients with osteoarthritis who present with a relatively high incidence of cardiovascular comorbidity, particularly hypertension, as has been shown[21].

We also found in this study that parecoxib produced total suppression of COX-2 mRNA (Fig. 2 B), and that protein expression is decreased by 30% in the aorta of normotensive rats (Fig. 3 B); this same drug did not induce changes in COX-1 mRNA levels (Fig. 2 A). These results were similar to those obtained with L-NAME, indicating that chronic suppression of NO synthase (NOS) may result in greater dependence on COX-2-derived PGI2 synthesis in terms of maintenance of vascular tone. Aspirin decreases the expression of the COX-1 protein, as well as COX-2, by 50 and 22%, respectively (Fig. 3 A and 3 B), the latter probably due to a 60% homology between the amino acids structure of COX-1 and COX-2. Aspirin binds to the residues of Ser 516 at the active site of COX-2, as well as to the residues of Ser 530 at the active site of COX-1, maintaining in apparent balance the products of the COX isoforms and their cardiovascular effects[22]. This may

explain the slight increase of MABP in our study (Fig. 1). In addition, a dynamic balance between the prostaglandins PGI2 and TXA2 (and many other mediators) is crucial in maintaining cardiovascular homeostasis and has critical pathophysiological and therapeutic implications[23]. Thus, some authors have found significant individual variations in the response to coxibs due to a number of candidate genes, including, in many individuals, *CYP2C9*, which is associated with a marked variability in the response to coxibs, although the importance of genetic variations with respect to cardiovascular risk remains unknown[24,25].

Some evidence from COX-2 inhibition trials has been published, suggesting that reduction in PGI2 may be associated with systemic hypertension in human subjects[26], exacerbating the TXA2 function[27]. At any rate, the results of our study showed that a decrease of COX-2 expression in aorta of rat by L-NAME could reflect the loss of positive regulation of NO in COX-2, a relationship previously demonstrated under several physiological and physiopathological conditions[19]. Evidence from randomized clinical trials to determine the safety of prescribing non-selective NSAID and COX-2 inhibitors in patients with high cardiovascular risk is extremely limited. In a study with parecoxib/valdecoxib, patients randomized to intravenous parecoxib/oral valdecoxib had a higher incidence of cardiovascular events than patients receiving placebo (2.0 vs. 0.5%; p = 0.03)[10]. To add to the controversies of the cardiovascular adverse effects of COX-2 inhibitors, several recent studies have shown that some COX-2 inhibitors are not associated with increased cardiovascular risk. The SUCCESS-I trial found no increased cardiovascular risks of celecoxib compared with diclofenac and naproxen in 13,274 patients with osteoarthritis. The TARGET trial found no significant difference in cardiovascular deaths between lumiracoxib and either ibuprofen or naproxen, irrespective of aspirin use, in 18,325 patients with osteoarthritis[20]. As with all of these drugs, the potential cardiovascular and gastrointestinal risks of their prescription needs to be weighed against the possible benefits for each individual patient and discussed with the patient. If the cardiovascular risk that increases with celecoxib is small and lower than that of most of the other NSAIDs, the concern would be of increasing complications in a patient with high cardiovascular risk if the patient were to be prescribed another NSAID[11,28]. Novel therapeutic strategies in hypertension aim at reversing endothelial dysfunction, which has been implicated in the pathogenesis and clinical course of hypertension and its cardiovascular complications[23].

In conclusion, our results suggest that the COX-2 inhibitor parecoxib increases the blood pressure of normotensive rats by suppression of COX-2 gene expression and reduced protein production that apparently induce cardiovascular lack of control of the arterial pressure.

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