Cyclooxygenase-2 Inhibitors Attenuate Angiotensin II–Induced Oxidative Stress, Hypertension, and Cardiac Hypertrophy in Rats

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Abstract—Angiotensin II is an important oxidative stress mediator. Our previous studies have indicated that the potent antioxidative properties of acetylsalicylic acid play an important role in its cardiovascular protective effects. There are some ongoing controversies concerning the use of selective cyclooxygenase-2 inhibitors in cardiovascular disease. The aim of this study was to determine whether the cyclooxygenase-2 selective inhibitors rofecoxib and nimesulide possess antioxidative and cardiovascular protective effects against angiotensin II. Chronic subcutaneous angiotensin II infusion increased cardiovascular but not colonic tissue superoxide production, heart/body weight ratio, and blood pressure. Moreover, angiotensin II selectively increased cardiac cyclooxygenase-2 but not cyclooxygenase-1 expression, which was totally prevented by acetylsalicylic acid treatment. Similar to acetylsalicylic acid, rofecoxib or nimesulide treatments significantly attenuated angiotensin II–induced oxidative stress, hypertension, and cardiac NAD(P)H oxidase subunit p47phox expression. Rofecoxib also reduced cardiac hypertrophy. Treatment with nonselective anti-inflammatory drugs ibuprofen, indomethacin, or salicylic acid did not show any effect on angiotensin II–induced superoxide production, hypertension, or cardiac hypertrophy. Although acetylsalicylic acid and salicylic acid inhibited angiotensin II–induced nuclear factor κB (NF-κB) activation, nimesulide did not modify NF-κB activation. In conclusion, cyclooxygenase-2 pathway is implicated in angiotensin II–induced oxidative stress and deleterious cardiovascular changes. Rofecoxib and nimesulide produced significant antioxidative effect by reducing NAD(P)H oxidase–dependent superoxide generation. These effects seem to be independent of NF-κB inhibition. (Hypertension. 2005;45:1139-1144.)

Key Words: cyclooxygenase ■ oxidative stress ■ angiotensin II ■ free radicals ■ hypertension, experimental

Excessive production of the superoxide anion (O$_2^-$) is associated with oxidative stress and subsequent cardiovascular tissue injury. Numerous clinical and experimental evidences indicate that oxidative stress plays an important pathogenetic role in cardiovascular diseases, including atherosclerosis and hypertension.

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase (COX) activity, which converts arachidonic acid to various prostaglandins and thromboxanes. This enzyme is found in 2 isoforms: the constitutive isoform, COX-1, and the inducible isoform, COX-2. There are 3 classes of COX inhibitors: aspirin (acetylsalicylic acid [ASA]), nonselective NSAIDs (eg, ibuprofen, indomethacin), and selective COX-2 inhibitors (eg, celecoxib, rofecoxib, and nimesulide). Our previous results indicated that ASA is a potent antioxidative agent, which effectively reduced cardiovascular tissue O$_2^-$ production, prevented or attenuated the development of hypertension, restored the impaired vasorelaxation in spontaneously hypertensive rats, and prevented angiotensin II (Ang II)–induced oxidative stress, hypertension, and free radicals and cardiovascular tissue hypertrophies. However, it was not clear whether other NSAIDs, especially the new selective COX-2 inhibitors, possessed similar antioxidative and cardiovascular protective properties as ASA and whether the antioxidative effect of NSAIDs could be observed in noncardiovascular tissues, such as colon.

Previous studies have indicated that Ang II increased cardiovascular tissue NAD(P)H oxidase activity and stimulated cardiovascular O$_2^-$ production. This oxidative mechanism is thought to play an important role in Ang II–mediated trophic cardiovascular changes, such as cardiovascular tissue hypertrophy and hypertension. Moreover, recent data also suggested that Ang II is an important inflammatory mediator, which increases COX-2 expression in cultured cells and activates nuclear factor κB (NF-κB) in cardiovascular and renal tissues.

The present study was designed to determine whether the COX-2 selective NSAIDs and the classic nonselective NSAIDs possess similar antioxidative effects as those of ASA in rats chronically infused with Ang II.
Methods

Animals
Studies were performed in male Sprague-Dawley rats (weighing 225 to 250 g; Charles River Laboratories; St-Constant, Canada). All experimental procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and monitored by an institutional care committee. Animals were given free access to drinking water, whereas for anti-inflammatory drug–treated rats, ASA (100 mg/kg per day), nimesulide (5 mg/kg per day), indomethacin (5 mg/kg per day), ibuprofen (100 mg/kg per day), or salicylic acid (120 mg/kg per day) was added into the drinking water. Rofecoxib (oral suspension Vioxx; 5 mg/kg per day) was given by gavage. For Ang II treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg IP), and osmotic pumps (model 2002; Alza Corp) were implanted subcutaneously in the flank region. Ang II was delivered by the pumps at a rate of 200 ng/kg per minute for 12 days. For simultaneous Ang II and anti-inflammatory drug treatment, the 2 treatments were started concomitantly. The systolic blood pressure (BP) was measured using tail-cuff plethysmography (Harvard Apparatus Ltd) before and every other day during the treatment.

At the end of the treatment, the thoracic aorta, the hearts, and the distal colon were excised and weighed. The heart/body weight ratio was calculated by using the whole ventricle weight (in milligrams) over the body weight (in grams).

Aortic Smooth Muscle Cell Culture and NF-κB Assay
Aortic smooth muscle cells (SMCs) from Sprague-Dawley rats were isolated and cultured as described previously. The cells were treated with vehicle (control) or Ang II (10–7 mol/L) alone or with 1 of the following NSAIDs: ASA (10–6 mol/L), nimesulide (4×10–5 mol/L), indomethacin (10–5 mol/L), ibuprofen (10–4 mol/L), salicylic acid (10–4 mol/L), or losartan (10–6 mol/L) for 48 hours. At the end of treatment, SMCs were scraped mechanically and washed twice by centrifugation. The nuclear extracts were prepared as described previously, and the activated NF-κB was measured with BIOXYTECH NF-κB Chemiluminescent Assay kit (OXISResearch).

O2− Measurement
O2− production was measured using the lucigenin-enhanced chemiluminescence method with low concentration (5 μmol/L) of lucigenin as described previously. To evaluate the intrinsic NAD(P)H oxidase activity, aortic rings were first incubated with NAD(P)H oxidase inhibitor diphenylene iodonium (DPI; 100 μmol/L) for 10 minutes at room temperature before O2− production was evaluated, and the DPI-inhibitable O2− production was calculated as the difference in O2− production observed in the presence or absence of DPI.

NAD(P)H Oxidase Subunit p47phox and COX Enzyme Expression Assay
Ventricular tissue was frozen and crushed in liquid nitrogen. Lysates and Western blots were performed as described previously with 20 μg of proteins loaded on gels. For COX assay, rabbit COX-1 or COX-2 polyclonal antibody (USBiological) was used. Membranes were exposed to Kodak X-Omat blue films for 2 minutes. Gels were scanned and densitometry of the bands was assessed using Scion image 4.0.2 (Scion). Results for each condition were divided by control values to estimate the magnitude of changes compared with controls.

Results
Results are expressed as mean±SEM, and statistical comparison was made by 1-way ANOVA.

Effects of Ang II on Cardiac Tissue COX-1 and COX-2 Expression In Vivo
Twelve days of Ang II infusion significantly increased the cardiac tissue COX-2 enzyme expression by 48% but did not modify the COX-1 enzyme expression (Figure 1). The increase in COX-2 expression was totally prevented by ASA, whereas ASA had no effect on COX-1 expression.

Effect of NSAIDs on Ang II–Induced Oxidative Stress
Ang II infusion importantly increased the aortic and cardiac tissue O2− production by 72% and 32% from basal values of 1678±76 and 233±14 cpm/mg tissue, respectively, but did not change the colon O2− production. Moreover, Ang II infusion also increased the aortic DPI-inhibitable O2− production by 55% from 1294±89 to 2006±162 cpm/mg tissue (P<0.05).

Similar to the effect of ASA, the concomitant treatment with rofecoxib in Ang II–infused rats effectively reduced the
Ang II–stimulated $O_2^{−}$ production by 34% and 28% in aorta and heart, respectively (Figure 3), and lowered the aortic DPI-inhibitable $O_2^{−}$ production by 42% to 1167±176 cpm/mg tissue. Moreover, the simultaneous nimesulide treatment also significantly reduced the Ang II–stimulated aortic and cardiac tissue $O_2^{−}$ production by 25% and 21% (Figure 3) and the aortic DPI-inhibitable $O_2^{−}$ production by 34% to 1317±84 cpm/mg tissue. In contrast, simultaneous indomethacin or ibuprofen treatments did not significantly reduce Ang II–stimulated $O_2^{−}$ production (Figure 3). Moreover, in a different experiment, the simultaneous salicylic acid treatment did not prevent the production of aortic superoxide production induced by Ang II (control 1275±158 cpm/mg tissue; Ang II 2076±252 cpm/mg tissue; Ang II +SA 2165±66 cpm/mg tissue).

Ang II infusion also increased by 3× the cardiac tissue expression of NAD(P)H oxidase subunit p47phox. Simultaneous ASA, rofecoxib, or nimesulide treatment attenuated significantly the Ang II–stimulated p47phox expression (Figure 4). Indomethacin but not ibuprofen treatment also slightly but significantly reduced Ang II–induced p47phox expression.

To determine the possible direct acute inhibition of these anti-inflammatory drugs on $O_2^{−}$ production, aortic rings were incubated in vitro with each NSAID for 10 minutes before the $O_2^{−}$ level was evaluated. None of the anti-inflammatory drugs modified the aortic tissue $O_2^{−}$ production. It is also noteworthy to observe that none of the anti-inflammatory drug oral treatments alone for 12 days modified the basal BP and heart/body weight ratio in normotensive control rats.

**Effect of NSAIDs on Ang II–Induced Hypertension and Cardiac Hypertrophy**

Chronic Ang II infusion progressively increased the BP from basal level of 142±2 to 201±5 mm Hg at the end of 12 days of infusion. Simultaneous ASA treatment completely abolished the BP elevation induced by the Ang II infusion, whereas rofecoxib or nimesulide treatment markedly attenuated the Ang II–induced hypertension and kept the BP at 154±4 and 172±4 mm Hg, respectively, at the end of 12 days of Ang II infusion (Figure 5A). In contrast, indomethacin, ibuprofen, or salicylic acid (data not shown) treatments did not show any significant effect on Ang II–induced hypertension.

Similar results were also observed on heart/body weight ratio (Figure 5B). Ang II infusion increased this ratio significantly from basal level of 2.23 to 2.75. In these animals, Ang II infusion induced a slight but not statistically significant decrease (−3%) of body weight growth (83±4 g during the 12 days of treatment) compared with control rats (92±9 g; $P=0.29$). The simultaneous ASA or rofecoxib treatment significantly inhibited the Ang II–induced cardiac hypertrophic effect without changing their body weight growth (83 g or 84 g, respectively). Nimesulide treatment slightly but not significantly inhibited the Ang II–induced cardiac hypertrophy (heart/body weight ratio 2.57; $P=0.62$). Indomethacin or ibuprofen treatment did not modify the Ang II–induced cardiac hypertrophy, and salicylic acid treatment did not modify this ratio as well (2.67 versus 2.71 in Ang II alone group).

**Effect of NSAIDs on Ang II–Induced NF-κB Activation**

Ang II treatment for 48 hours increased NF-κB activation by 74%, from basal level of 1685±267 to 2925±270 pg/μg
nuclear protein. This effect of Ang II was partially blocked by concomitant treatment with losartan. Simultaneous treatment with ASA or salicylic acid, but not with nimesulide, indomethacin, or ibuprofen, also significantly inhibited the Ang II–induced NF-κB activation (Figure 6).

Discussion

As was shown with ASA, the present results indicated that selective COX-2 inhibition by rofecoxib and nimesulide significantly attenuated Ang II–induced oxidative stress through reducing the NAD(P)H oxidase activity, as reflected by the decrease in the DPI-inhibitable O$_2^\cdot$ production and in the expression of NAD(P)H oxidase subunit p47$^{\text{phox}}$. The antioxidative effects of rofecoxib and nimesulide, which were less potent than that of ASA, were also associated with a significant attenuation of Ang II–induced hypertension, which was also less marked than the effects of ASA. Rofecoxib also attenuated the Ang II–induced cardiac hypertrophy. In contrast, treatment with nonselective NSAIDs indomethacin, ibuprofen, and even salicylic acid did not modify the Ang II–induced oxidative stress and cardiovascular alterations.

This is the first report showing that rofecoxib possesses antioxidative property and inhibits Ang II–induced cardiovascular alterations. Recent data indicated that selective COX-2 inhibition by celecoxib reduces oxidized LDL in patients with coronary artery disease,15 slightly decreased salt-induced hypertension, and normalized the indicator of oxidative stress, 8-isoprostane, in hypertensive rats.16 However, different from our result, rofecoxib (2 mg/kg per day) treatment did not show any antioxidative effect in those salt-induced hypertensive rats. This difference could be attributable to the low rofecoxib dose used in that study. The antioxidative effect of nimesulide was reported previously in human neutrophils17 and in rat colonic mucosa.18 Here, our result

Figure 4. Effect of Ang II and NSAIDs on cardiac tissue NAD(P)H oxidase subunit p47$^{\text{phox}}$ expression evaluated by Western blot. Rats were treated with Ang II alone (Ang II; n=5) or concomitant with ASA (Ang II+ASA; n=7), rofecoxib (Ang II+Rof; n=5), nimesulide (Ang II+Nim; n=5), indomethacin (Ang II+Indo; n=5), or ibuprofen (Ang II+Ibu; n=5). The top shows a representative result. \( + \cdot P<0.01 \text{ vs control, } ^* P<0.05 \text{ and } ^{**} P<0.01 \text{ vs Ang II alone.} \)

Figure 5. Development of BP (A) and heart/body weight ratio (B) from rats treated with Ang II alone or concomitant with ASA, rofecoxib (Rof), nimesulide (Nim), indomethacin (Indo) or ibuprofen (Ibu). \( + \cdot P<0.01 \text{ vs control, } ^* P<0.05 \text{ and } ^{**} P<0.01 \text{ vs Ang II alone.} \)

Figure 6. NF-κB activation obtained from 6 separate experiments on cultured aortic SMCs treated with Ang II alone or concomitant with losartan (Los), ASA, ibuprofen (Ibu), nimesulide (Nim), indomethacin (Indo) or salicylic acid (SA). \( + \cdot P<0.01 \text{ vs control, } ^* P<0.05 \text{ and } ^{**} P<0.01 \text{ vs Ang II alone.} \)
showed that nimesulide is also an antioxidative agent in cardiovascular tissues.

Although the inhibition of the 2 COX isoforms and the anti-inflammation by indomethacin and ibuprofen is much more potent than ASA and is at least as potent as rofecoxib and nimesulide, the treatment with indomethacin and ibuprofen did not reduce the cardiovascular tissue $O_2^{−}$ level. Thus, the differences in antioxidative property between different NSAIDs could not be explained by their inhibitory potency on COX enzyme or by their anti-inflammatory effects. Because the COX-2 selective inhibitors rofecoxib, nimesulide, and celecoxib are structurally different molecules and because all of them have been demonstrated to possess antioxidative properties, it may be postulated that the selective COX-2 inhibition might be involved in the antioxidative effect of these drugs.

Although there is no direct evidence demonstrating a causal relationship between COX enzyme activation and oxidative status, some studies have provided suggestions indicating a positive role of COX-2 in the induction of oxidative stress. It is known that COX-2 is a key mediator of inflammation and that inflammatory reaction produces local or systemic oxidative stress. Thus, inhibition of COX-2 activity is expected to reduce the COX-2–dependent oxidative stress. However, little is known about the role of COX-1 in redox status. Because our results showed that only COX-2 selective but not COX nonselective inhibitor reduces the $O_2^{−}$ production, it may be postulated that COX-1 activation might have an opposite effect of that of COX-2 on oxidative stress.

Many studies have demonstrated that Ang II is an important oxidative stress mediator. Our present results showed that Ang II–stimulated $O_2^{−}$ generation was observed only in cardiovascular tissues but not in colon, although previous studies have demonstrated the presence and the functions of AT1 and AT2 receptors in colon. Our results have also shown that Ang II selectively stimulated cardiac COX-2 but not COX-1 enzyme expression in vivo. Similar result was also reported by Young et al on cultured SMCs. Interestingly, our data also showed that ASA prevented the Ang II–induced COX-2 expression, suggesting a possible link between COX-2 expression and the oxidative stress and hypertension induced by Ang II. However, the protective effects of the selective COX-2 inhibitors against Ang II–induced cardiovascular changes observed in the present study were not mediated by their anti-inflammatory effect but rather by their antioxidative properties through reducing the NAD(P)H oxidase–dependent $O_2^{−}$ production because the nonantioxidative NSAIDs such as ibuprofen did not show any effect.

The NF-κB has been shown to be a key element in the response of cells to inflammatory stimuli. Chronic inflammation has been postulated to participate in the development of cardiovascular diseases, and recent data have shown that Ang II is an important proinflammatory mediator. Thus, it is assumed that inflammation may play an important role in Ang II–induced cardiovascular alteration. Indeed, experimental studies have shown that Ang II infusion elicited the activation of NF-κB in rat aortic and renal tissues, which was associated with inflammatory cell infiltration and tissue damage. However, it is not known whether the NF-κB pathway activation plays a role in Ang II–induced oxidative stress and cardiovascular alterations.

Our studies have confirmed that Ang II is a strong activator of NF-κB in vascular SMCs. The Ang II–induced NF-κB activation was prevented by concomitant treatment with ASA or salicylic acid but not by the other NSAIDs. Moreover, Ang II–induced oxidative stress and hypertension were prevented or attenuated by ASA, rofecoxib, or nimesulide, whereas ASA and rofecoxib also inhibited the Ang II–induced cardiac hypertrophy. In contrast, salicylic acid, which also inhibited NF-κB and the other anti-inflammatory drugs indomethacin or ibuprofen, did not prevent the oxidative stress, the hypertension, or cardiac hypertrophy induced by Ang II. Therefore, the Ang II–stimulated NF-κB activation, as well as the inflammatory process, can be dissociated from Ang II–induced oxidative stress, hypertension, and cardiac hypertrophy. Thus, these results strongly suggest that the Ang II–elicited NF-κB activation and inflammatory reaction do not play an important role in Ang II–induced oxidative stress and cardiovascular alterations in our animal model.

It is well known that Ang II can promote cardiac hypertrophy through BP-independent mechanisms and that oxidative stress could play an important role in this effect. On the other hand, BP change can also influence the cardiac mass. Therefore, the antihypertrophic effects of ASA and rofecoxib are apparently related to their capacity to prevent the development of hypertension. However, the significantly less potent hypotensive effect of nimesulide, which was probably attributable to its relatively lesser effectiveness in reducing Ang II–induced vascular oxidative stress, could explain the nonsignificant effect of this drug on Ang II–induced cardiac hypertrophy.

Theoretically, the selective COX-2 inhibition alters the balance of vasoactive eicosanoids (thromboxane and prostacyclin) and favors thrombosis and ischemic events. This hypothesis seems to be supported by secondary analyses of phase III clinical trials and by the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial. Clinical data also indicated that use of a selective COX-2 inhibitor, especially rofecoxib, is associated with a significant increase in systolic BP, more notably in hypertensive patients.

Our results indicated that selective COX-2 inhibitors rofecoxib and nimesulide reduced superoxide production and prevented or attenuated Ang II–induced oxidative stress and hypertension, and that rofecoxib also reduced Ang II–induced cardiac hypertrophy. Thus, rofecoxib and nimesulide have the potential to exert a beneficial impact outcome in cardiovascular patients with oxidative stress and enhanced Ang II activity.

**Perspectives**

The present results reveal a relationship between the COX-2 pathway activation and local tissue superoxide production and also indicate that COX-2 is implicated importantly in Ang II–induced oxidative stress and deleterious cardiovascular changes. Thus, the selective inhibition of the COX-2 pathway provides a potentially important therapeutic target for treating oxidative stress–mediated diseases.
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