Cyclooxygenase 2-Selective and Nonselective Nonsteroidal Anti-Inflammatory Drugs Induce Oxidative Stress by Up-Regulating Vascular NADPH Oxidases

Huige Li, Marcus Hortmann, Andreas Daiber, Matthias Oelze, Mir Abolfazl Ostad, Petra M. Schwarz, Hui Xu, Ning Xia, Andrei L. Kleschyov, Christian Mang, Ascan Warnholtz, Thomas Münzel, and Ulrich Förstermann

Departments of Pharmacology (H.L., M.H., P.M.S., H.X., N.X., C.M., U.F.) and Internal Medicine II (A.D., M.O., M.A.O., A.L.K., A.W., T.M.), Johannes Gutenberg University, Mainz, Germany

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ABSTRACT

Cyclooxygenase 2-selective inhibitors (coxibs) and nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with an increase in cardiovascular events. The current study was designed to test the effect of coxibs and nonselective NSAIDs on vascular superoxide and nitric oxide (NO) production. mRNA expression of endothelial NO synthase (eNOS) and of the vascular NADPH oxidases was studied in spontaneously hypertensive rats (SHR) and in human endothelial cells. The expression of Nox1, Nox2, Nox4, and p22phox was increased markedly by the nonselective NSAIDs diclofenac or naproxen and moderately by rofecoxib or celecoxib in the aorta and heart of SHR. The up-regulation of NADPH oxidases by NSAIDs was associated with increased superoxide content in aorta and heart, which could be prevented by the NADPH oxidase inhibitor apocynin. NSAIDs reduced plasma nitrite and diminished the phosphorylation of vasodilator-stimulated phosphoprotein. This demonstrates a reduction in vascular NO production. Aortas from diclofenac-treated SHR showed an enhanced protein nitrotyrosine accumulation, indicative of vascular peroxynitrite formation. Peroxynitrite can uncouple oxygen reduction from NO synthesis in eNOS. Accordingly, the eNOS inhibitor N\(\text{G}\)-nitro-L-arginine methyl ester reduced superoxide content in aortas of NSAID-treated animals, demonstrating eNOS uncoupling under those conditions. Also in human endothelial cells, NSAIDs increased Nox2 expression and diminished production of bioactive NO. In healthy volunteers, NSAID treatment reduced nitroglycerin-induced, NO-mediated vasodilatation of the brachial artery. These results indicate that NSAIDs may increase cardiovascular risk by inducing oxidative stress in the vasculature, with nonselective NSAIDs being even more critical than coxibs in this respect.

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the activity of cyclooxygenase (COX). COX is the pacemaker enzyme in the synthesis of prostaglandins and thromboxane from arachidonic acid. Two isoforms of COX have been identified; COX1 is constitutively expressed in many tissues and generates prostanoids mediating normal physiological functions. COX2, on the other hand, is induced at sites of inflammation, but it is also expressed in response to physiologic stimuli (see below). Aspirin and other nonselective NSAIDs inhibit both COX isoenzymes. COX2-selective inhibitors (coxibs) were developed to provide the anti-inflammatory and analgesic effects of NSAIDs with reduced gastrointestinal toxicity (Antman et al., 2005). However, serious cardiovascular side effects have been reported for coxibs, including rofecoxib, celecoxib, parecoxib, and valdecoxib (Bresalier et al., 2005; Nussmeier et al., 2005; Solomon et al., 2005). Compared with placebo, allocation to a coxib is associated with a 42% relative increase in the inci-
dence of total vascular events (1.2%/year versus 0.9%/year), mainly attributable to an increased risk of myocardial infarction (0.6%/year versus 0.3%/year), with little apparent difference in other vascular outcomes: stroke (0.4%/year versus 0.4%/year) and vascular death (coxib 0.3%/year versus placebo 0.2%/year) (Kearney et al., 2006). The most widely accepted hypothesis to explain the greater numbers of cardiovascular events associated with coxibs is that these agents cause an imbalance between vascular prostacyclin and platelet thromboxane (Antman et al., 2005). Platelets are without COX2 and produce thromboxane via COX1. Endothelial cells express COX1, and the shear stress of the flowing blood stimulates COX2 expression (Inoue et al., 2002). In fact, COX2 is the dominant source of prostacyclin from endothelial cells in vivo. Thus, according to the prostanooid imbalance theory, COX2 inhibition by coxibs would markedly reduce prostacyclin production from endothelial cells without inhibiting COX1 in platelets, and thus lead to a thromboxane overproduction and promote platelet-dependent thrombosis (Antman et al., 2005).

However, there is an increasing body of evidence indicating that this paradigm may be too simplistic and cannot explain the available clinical data (Hermann et al., 2005). For example, addition of aspirin (a preferential COX1 inhibitor) does not prevent the adverse cardiovascular effects of coxibs (Nussmeier et al., 2005). Moreover, also nonselective NSAIDs, which inhibit primarily COX1, have been found to be associated with an increased rate of cardiovascular events (Hippisley-Cox and Coupland, 2005; Kearney et al., 2006; McGgettigan and Henry, 2006).

Thus, prostanooid imbalance cannot explain all of the adverse cardiovascular effects of coxibs and traditional NSAIDs observed in clinical studies, and other pathogenic mechanisms have to be postulated.

Over the last years, evidence has accumulated showing that oxidative stress plays an important role in the pathogenesis of cardiovascular disease (Griendling and Fitz-Gerald, 2003; Förstermann and Münzel, 2006), and there are some hints that NSAIDs may affect the redox status of vascular tissues. For example, rofecoxib has been shown to increase the susceptibility of human low-density lipoprotein and cell membrane lipids to oxidative modification (Walter et al., 2004). Rofecoxib can also promote nonenzymatic formation of isoprostanes from biological lipids (Mason et al., 2007). In contrast, in salt-sensitive Dahl rats fed a high-sodium diet, elevated levels of 8-isoprostane and endothelial dysfunction were normalized by celecoxib and not affected by rofecoxib or diclofenac (Hermann et al., 2003).

Therefore, the current study was designed to investigate whether NSAIDs induce or reduce vascular oxidative stress, and if so, by which mechanism. Cardiovascular side effects of NSAIDs are being observed more frequently in patients with pre-existing cardiovascular disease (Bennett et al., 2005). Patients with hypertension, dyslipidemia, diabetes mellitus, congestive heart failure, or chronic renal disease seem to have a significantly higher risk associated with the use of NSAIDs compared with patients without these conditions (Huang et al., 2006). For this reason, we performed our experiments in an animal model of cardiovascular disease, the spontaneously hypertensive rat (SHR). Similar to human pathology, these animals show an enhanced production of reactive oxygen species (ROS) in their vasculature (Nabha et al., 2005).

Materials and Methods

Animals and Treatment with NSAIDs. Male SHR (6 months of age) were obtained from Charles River Laboratories (Sulzfeld, Germany) and assigned randomly to receive rofecoxib [4-(4-methylsulfonylphenyl)-3-phenyl-5H-furan-2-one, CAS number 162011-90-7, chemical formula C14H11Cl2NO2, 10 mg/kg/day] (MSD Sharp & Doehme, Munich, Germany; obtained before the withdrawal in November 2004), celecoxib [4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide, CAS number 169590-42-5, chemical formula C21H14F4N2O3S, 30 mg/kg/day] (Pfizer, Karlsruhe, Germany), diclofenac [2-(2,6-dichlorophenylaminomethyl)phenylacetic acid, CAS number 153378-56-5, chemical formula C10H11ClN2O2, 5 mg/kg/day] (Sigma-Aldrich, Deisenhofen, Germany), or naproxen [(1R,2S)-2-(6-methoxynaphthalen-2-yl)propanoic acid, CAS number 22204-53-1, chemical formula C14H11Cl2NO5, 50 mg/kg/day] (Sigma-Aldrich) mixed into the chow for 2 weeks. The doses of the NSAIDs were extrapolated from doses applied to humans. COX1 activity was not affected at the doses selected for rofecoxib (Höcherl et al., 2002) and celecoxib (Kitahara et al., 2002). Apocynin [1-(4-hydroxy-3-methoxyphenyl)ethanone, CAS number 498-02-2, chemical formula C10H9NO, (Sigma-Aldrich), 70 mg/kg/day], a selective NADPH oxidase inhibitor, was given in drinking water. The use of animals in this study complies with the Institute of Laboratory Animal Resources (1996) and the German law on the protection of animals. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Real-Time Reverse Transcription-Polymerase Chain Reaction for mRNA Expression of NADPH Oxidase Subunits and Endothelial Nitric-Oxide Synthase. mRNA expression of endothelial nitric-oxide synthase (eNOS), Nox isoforms of NADPH oxidase (Nox1, Nox2, and Nox4), and NADPH oxidase subunit p22phox was analyzed with quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) using an iCyclerTM iQ System (Bio-Rad Laboratories, Munich, Germany), as described previously (Li et al., 2006). Total RNA was isolated from aorta and heart of SHR using an RNeasy Fibrous Tissue Kit (QIAGEN, Hilden, Germany). Five hundred nanograms of total RNA were used for real-time RT-PCR analysis with the Quantitect Probe RT-PCR kit (QIAGEN). Gene expression of the target genes was normalized to the endogenous control, glyceraldehyde 3-phosphate dehydrogenase mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.

Measurement of Superoxide Concentrations in Heart Membrane Fractions. Rat heart tissues were homogenized (glass/glass) in buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and centrifuged at 2000 rpm for 5 min at room temperature. The supernatant was removed and centrifuged at 20,000 rpm for 20 min at 4°C. Again the supernatant was removed and centrifuged at 100,000 rpm for 60 min at 4°C. The pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors, and further diluted in phosphate-buffered saline to give a final protein concentration of 0.7 mg/ml and a final dithiothreitol concentration of 200 μM. The lucigenin (5 μM)-derived chemiluminescence of the membrane suspensions was detected in a Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany) in the presence of 200 μM NADPH (Daiber et al., 2005). The activity was not affected at the doses selected for rofecoxib (Höcherl et al., 2002) and celecoxib (Kitahara et al., 2002). Apocynin [1-(4-hydroxy-3-methoxyphenyl)ethanone, CAS number 498-02-2, chemical formula C10H9NO, (Sigma-Aldrich), 70 mg/kg/day], a selective NADPH oxidase inhibitor, was given in drinking water. The use of animals in this study complies with the Institute of Laboratory Animal Resources (1996) and the German law on the protection of animals. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Measurement of Aortic Superoxide Concentrations by Lucigenin Chemiluminescence. Aortas from SHR were cut into 3-mm rings and equilibrated in a Krebs-HEPES buffer for 30 min at 37°C, with or without the nitric-oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (1-NAME; 1 mM). Then, lucigenin (5 μM)-derived chemiluminescence was detected in a Lumat LB 9507.
were observed for any of the laboratory parameters before and after NSAID treatment.

Measurement of Aortic ROS Content by L-012 Chemiluminescence. Aorta rings at 3-mm length were incubated for 30 min at 37°C in 96-well plates in Hanks' balanced salt solution (PAA Laboratories, Cölbe, Germany) containing 100 µM of the luminal derivative L-012 (Daiber et al., 2004; Li et al., 2006). L-012-derived chemiluminescence was measured using a Microplate Luminometer (Berthold Technologies). The photon counts were normalized for the dry weight of aortic tissue.

Measurement of ROS Content by Dibydroethidium Fluorescence. The oxidative fluorescent dye dibydroethidium (DHE) was used to evaluate the amount of superoxide in situ, as described previously (Li et al., 2006). Frozen sections (10 µm) from aorta or heart were incubated with 10 µM DHE. Photographs were taken using a fluorescence microscope at an excitation wavelength of 520 nm and an emission wavelength of 610 nm. To analyze the potential contribution of eNOS to ROS production, some sections were preincubated with l-NAME (1 mM) for 30 min (Li et al., 2006).

Plasma Total Peroxide As a Marker of Oxidative Stress. Plasma total peroxide concentrations were determined photometrically in EDTA plasma using a PerOx assay kit (Immundiagnostik, Bensheim, Germany), which is based on the reaction of horseradish peroxidase with plasma peroxides using tetramethylebenzidine as a chromogen substrate (450-nm wavelength) (Hildebrandt et al., 2002).

Western Blot Analysis. Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After blocking, immunoblotting was performed with a mouse monoclonal phosphorylated vasodilator-stimulated phosphorylation (P-VASP) phosphoserine 239 antibody (clone 16C2) (Merck, Bensheim, Germany), which is based on the reaction of horseradish peroxidase with plasma peroxides using tetramethylebenzidine as a chromogen substrate (450-nm wavelength) (Hildebrandt et al., 2002).

Detection of Nitrotyrosine in Aortic Sections. Frozen sections of aorta (10 µm) were incubated with a rabbit polyclonal anti-nitrotyrosine antibody (catalog no. 06-284; Millipore Bioscience Reagents, Temecula, CA) at a dilution of 1:100. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was used as secondary antibody. After addition of avidin/biotinylated enzyme (peroxidase) complex (Vectastain ABC kit; Vector Laboratories), the chromogen substrate (450-nm wavelength) (Hildebrandt et al., 2002).

NSAIDs enhance the expression of NADPH oxidases in aorta and heart of SHR. Treatment of SHR with rofecoxib (10 mg/kg/day), celecoxib (30 mg/kg/day), diclofenac (5 mg/kg/day), or naproxen (50 mg/kg/day) for 2 weeks resulted in enhanced mRNA expression of NADPH oxidases in RFL-6 samples.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td><strong>Laboratory parameters of the 10 volunteers receiving rofecoxib (50 mg once daily), celecoxib (200 mg twice daily), or diclofenac (75 mg twice daily), each for 5 days</strong></td>
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before Placebo</th>
<th>After Placebo</th>
<th>Before Rofecoxib</th>
<th>After Rofecoxib</th>
<th>Before Celecoxib</th>
<th>After Celecoxib</th>
<th>Before Diclofenac</th>
<th>After Diclofenac</th>
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<tr>
<td>Total cholesterol</td>
<td>155.1 ± 5.8</td>
<td>150.3 ± 7.4</td>
<td>158.5 ± 11.4</td>
<td>147.6 ± 10.7</td>
<td>158.6 ± 11.8</td>
<td>154.0 ± 9.2</td>
<td>152.9 ± 11.4</td>
<td>156.5 ± 9.1</td>
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<tr>
<td>Low-density lipoprotein cholesterol</td>
<td>82.2 ± 9.1</td>
<td>84.4 ± 8.8</td>
<td>79.4 ± 10.4</td>
<td>75.1 ± 10.5</td>
<td>85.9 ± 10.0</td>
<td>81.0 ± 8.6</td>
<td>83.1 ± 9.4</td>
<td>84.4 ± 8.6</td>
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<tr>
<td>High-density lipoprotein cholesterol</td>
<td>54.1 ± 3.8</td>
<td>55.2 ± 3.3</td>
<td>55.5 ± 4.1</td>
<td>55.3 ± 3.6</td>
<td>53.6 ± 3.2</td>
<td>52.7 ± 3.2</td>
<td>53.6 ± 3.2</td>
<td>51.7 ± 2.9</td>
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<td>Glucose (mg/dl)</td>
<td>89.9 ± 4.3</td>
<td>89.0 ± 3.0</td>
<td>87.4 ± 3.3</td>
<td>89.3 ± 3.5</td>
<td>90.0 ± 3.3</td>
<td>88.6 ± 3.4</td>
<td>87.8 ± 1.4</td>
<td>88.7 ± 2.3</td>
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<tr>
<td>Brachial arterial diameter (mm)</td>
<td>4.07 ± 0.20</td>
<td>4.09 ± 0.22</td>
<td>4.01 ± 0.22</td>
<td>4.20 ± 0.17</td>
<td>4.12 ± 0.22</td>
<td>4.14 ± 0.25</td>
<td>4.08 ± 0.22</td>
<td>4.07 ± 0.18</td>
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The volunteers had the following baseline clinical characteristics: age, 35 ± 3 years; male, 50%; body mass index, 23.0 ± 0.9 kg/m²; hypertriglyceridemia, 20%; hypertension, 0%; diabetes, 0%; smoker, 10%. All measured laboratory parameters were normal for all volunteers. Data are expressed as mean ± S.E.M.; n = 7–10; no significant differences were observed for any of the laboratory parameters before and after NSAID treatment.
the aorta (Fig. 1A) as well as in the heart (Fig. 1B). In the aorta and in the heart, a marked increase of Nox1, Nox2, and Nox4 was seen with the nonselective NSAIDs diclofenac and naproxen. Rofecoxib and celecoxib also increased the expression of these Nox isoforms, but to a lesser extent. p22phox was significantly increased by diclofenac and naproxen, whereas rofecoxib and celecoxib had little (heart) or no effect (aorta).

**NSAIDs Induce Oxidative Stress in SHR.** Treatment of SHR with NSAIDs resulted in increased vascular ROS content as determined with DHE staining (Fig. 2A) and L-012 chemiluminescence (Fig. 2C). In accordance with the effects of NSAIDs on the expression of NADPH oxidases (Fig. 1), diclofenac and naproxen were more efficacious than rofecoxib or celecoxib at enhancing the superoxide content of the aorta from treated SHR. ROS content in heart was also increased by NSAIDs, as measured by DHE staining (Fig. 2B) and by lucigenin chemiluminescence (Fig. 2D). In agreement with the enhanced oxidative stress in cardiovascular tissues, plasma concentrations of total peroxides tended to be higher than control as determined with DHE staining (Fig. 2, A and C) as well as elevated plasma peroxides (at least for diclofenac; Fig. 2E). The increased superoxide concentrations in diclofenac-treated animals were reduced by the NOS inhibitor L-NAME, as determined with L-012 or lucigenin chemiluminescence (Fig. 5, A and B). Aortas from diclofenac-treated SHR showed a reduced cGMP content, which could be reversed by the NOS cofactor BH₄ (Fig. 5C). In diclofenac-treated SHR, an enhanced accumulation of protein nitrotyrosine was observed in the aorta, which was prevented by apocynin (Fig. 5D).

**NSAIDs Decrease the Level of Bioactive NO, but Up-Regulate eNOS Expression.** Treatment with NSAIDs decreased the plasma levels of nitrite, the oxidation product of NO (Fig. 4A). However, eNOS expression was not reduced but was even up-regulated by diclofenac or naproxen (Fig. 4B). In the aorta, vasodilator-stimulated phosphoprotein (VASP) phosphorylation was decreased by treatment with NSAIDs (Fig. 4, C and D), indicating a reduction in the levels of bioactive NO. Also in this respect, diclofenac and naproxen showed stronger effects than rofecoxib and celecoxib.

The increased superoxide concentrations in diclofenac-treated animals were reduced by the NOS inhibitor L-NAME. As determined with L-012 or lucigenin chemiluminescence (Fig. 5, A and B), Aortas from diclofenac-treated SHR showed a reduced cGMP content, which could be reversed by the NOS cofactor BH₄ (Fig. 5C). In diclofenac-treated SHR, an enhanced accumulation of protein nitrotyrosine was observed in the aorta, which was prevented by apocynin (Fig. 5D).

**NSAIDs Increase Nox2 Expression and Decrease the Level of Bioactive NO in Cultured Human Endothelial Cells.** Nox2 mRNA expression was increased in human EA.hy 926 endothelial cells by rofecoxib or diclofenac but not by celecoxib (10 μM each, 24 h; Fig. 6A).

When RFL-6 reporter cells were treated with conditioned media from nonstimulated EA.hy 926 endothelial cells, a basal cGMP level of 2.2 ± 0.1 pmol/10⁶ cells was measured. The cGMP level increased to 33.7 ± 1.5 pmol/10⁶ cells, when endothelial cells were stimulated with 300 μM ATP (Fig. 6B). This increase could be totally prevented with the NOS inhibitor L-NAME (1 mM), which reduced the cGMP level to 2.3 ± 0.2 pmol/10⁶ cells (data not shown). Pretreatment of EA.hy 926 cells for 24 h with rofecoxib or diclofenac significantly reduced cGMP levels in the RFL-6 reporter cell assay; a 24-h treatment with celecoxib only showed a tendency for reduced cGMP levels (Fig. 6B).

**NSAIDs Impair Nitroglycerin-Induced Dilatation of the Human Brachial Artery.** Healthy volunteers received the NSAIDs rofecoxib, celecoxib, or diclofenac at the highest recommended daily dose for 5 days (Fig. 7). Before and after the 5-day treatment with one of the NSAIDs, nitroglycerin-induced (NO-mediated) dilatation of the right brachial artery was measured noninvasively using high-resolution vascular ultrasound. All three NSAIDs impaired nitroglycerin-induced dilatation significantly (Fig. 7).

**Discussion**

The present study provides evidence in favor of a novel theory explaining the adverse cardiovascular effects of coxibs and nonselective NSAIDs. Our data demonstrate that both COX2-selective coxibs (rofecoxib and celecoxib) and, to an even greater extent, nonselective NSAIDs (diclofenac and naproxen) induce oxidative stress in the cardiovascular system of spontaneously hypertensive rats. This is indicated by enhanced vascular superoxide content (Fig. 2, A and C) as well as elevated plasma peroxides (at least for diclofenac; Fig. 2E). The increased superoxide content is consistent with an enhanced expression of NADPH oxidases in aorta and heart (Fig. 1). Treatment with the NADPH oxidase inhibitor apocynin prevented the enhanced vascular superoxide production by diclofenac (Fig. 3). In addition, an “uncoupled,” dysfunctional eNOS (see below) can contribute to oxidative stress.

Plasma nitrite was decreased in NSAID-treated animals (Fig. 4A), pointing to a diminished synthesis of vascular NO.
In principle, this could be the result of a decreased expression of eNOS. However, as demonstrated in Fig. 4B, eNOS mRNA expression was not reduced; diclofenac and naproxen even (paradoxically) up-regulated eNOS mRNA expression. The increased expression of eNOS mRNA is probably a consequence of an increased production of the reactive oxygen species H$_2$O$_2$. H$_2$O$_2$, the dismutation product of superoxide, has been shown to increase eNOS expression at the transcriptional and post-transcriptional levels (Drummond et al., 2000). In accordance with this hypothesis, the NSAIDs producing the most oxidative stress (diclofenac and naproxen; Fig. 2) were the ones that produced the greatest up-regulation of eNOS expression (Fig. 4B).

An involvement of the inducible NO synthase (iNOS) in the NSAID-induced reduction in plasma nitrite seems unlikely. It has been demonstrated that iNOS is expressed in aorta and heart of SHR (Vaziri et al., 2000). However, NSAIDs (diclofenac or celecoxib) did not modify iNOS expression or plasma nitrite/nitrate levels in a rat model (Hamilton and Warner, 1998). Thus, the changes in plasma nitrite in response to NSAIDs observed in our study probably reflect NO production from eNOS.

Our data further indicate that the decreased NO production in response to NSAIDs can be explained by a loss of eNOS enzyme activity or functionality. Enhanced oxidative stress can lead to an uncoupling of oxygen reduction from NO synthesis in eNOS ( Förstermann and Münzel, 2006). An uncoupled eNOS will produce superoxide at the expense of NO. We and others have shown previously that in vascular diseases associated with oxidative stress, eNOS is also up-regulated but dysfunctional. Thus, the NSAIDs are likely to produce oxidative stress and eNOS uncoupling similar to that observed in animal models of cardiovascular disease such as hypertension, hyperlipidemia, and diabetes mellitus ( Förstermann and Münzel, 2006).

Indeed, the increased superoxide content in diclofenac-treated SHR could be reduced by the NOS inhibitor L-NAME (Fig. 5, A and B), indicating that eNOS was in a dysfunctional state under these conditions. In many cardiovascular disease states associated with eNOS uncoupling, oxidative stress increases, which can lead to further eNOS dysregulation and NO deficiency. This vicious cycle exacerbates the underlying disease processes, reinforcing the importance of targeting the NO-eNOS pathway in cardiovascular medicine.
degradation of the NOS cofactor BH$_4$ is probably the common cause of eNOS dysfunction ( Förstermann and Münzel, 2006). This seems to be true also in NSAID-induced eNOS uncoupling, because substitution with BH$_4$ restored eNOS functionality (Fig. 5C). It is important to note that not all ROS are able to oxidize BH$_4$. Particularly peroxynitrite—the direct reaction product of NO and superoxide—is able to do so ( Förstermann and Münzel, 2006). Peroxynitrite also causes tyrosine nitration of proteins, and, indeed, NSAIDs led to an enhanced nitrotyrosine accumulation (Fig. 5D).

Additional evidence for eNOS uncoupling after NSAID administration came from the demonstration of a reduced VASP phosphorylation (Fig. 4, C and D) in the aorta. This indicates a functional impairment of the NO/cGMP/cGMP-dependent protein kinase (cGK) pathway (most probably caused by the diminished formation of bioactive NO). VASP is a validated substrate of cGMP-dependent protein kinase (cGK), and P-VASP can be used as an indicator of the integrity of the NO/cGMP/cGK pathway ( Oelze et al., 2000). The reduction in P-VASP was more pronounced with the nonselective NSAIDs than with coxibs.

When exposing human endothelial cells to COX inhibitors, rofecoxib and diclofenac increased Nox2 expression and reduced cGMP (the second messenger generated in response to bioactive NO) significantly, whereas celecoxib did not (Fig. 6, A and B). This result is interesting in view of clinical trials demonstrating a more favorable cardiovascular risk profile of celecoxib relative to rofecoxib ( McGettigan and Henry, 2006). In an animal study, celecoxib even improved endothelial function, whereas rofecoxib did not ( Hermann et al., 2003). Different intrinsic physicochemical properties of celecoxib (sulfonamide) and rofecoxib (sulfone) may be responsible (in part) for the difference in cardiovascular effects of the two coxibs ( Walter et al., 2004; Mason et al., 2007).

It is established that coxibs and nonselective NSAIDs can produce increases in blood pressure in rats ( Höcherl et al., 2002; Hermann et al., 2003) and in humans ( Mukherjee et al., 2001).
Fig. 4. NSAIDs decrease NO bioactivity, increase eNOS expression, and reduce VASP phosphorylation. SHR were treated with rofecoxib (10 mg/kg/day), celecoxib (30 mg/kg/day), diclofenac (5 mg/kg/day), or naproxen (50 mg/kg/day) for 2 weeks. A, plasma nitrite was determined by ozone chemiluminescence. B, eNOS mRNA expression in aorta was analyzed with quantitative real-time RT-PCR. C and D, VASP phosphorylation in the aorta was analyzed with Western blot. α-Actinin was determined for normalization. The blot shown is representative of three independent experiments performed in duplicates. Columns represent mean ± S.E.M., n = 3–12 (*, P < 0.05, **, P < 0.01, and ***, P < 0.001, compared with control).

Fig. 5. Diclofenac induces eNOS uncoupling in SHR aortas and leads to tyrosine nitration (indicative of peroxynitrite formation). A to C, SHR were treated for 2 weeks with vehicle (Ctr) or diclofenac (Dic; 5 mg/kg/day). Superoxide content in aortic rings was determined with L-012 (A) or lucigenin (Luc.) chemiluminescence (B) in the absence or presence of the NOS inhibitor L-NAME (L-N; 1 mM). C, aortic rings were pretreated with BH4 and then stimulated with 300 nM acetylcholine. The cGMP content in the aortic tissues was determined with radioimmunoassay. Columns represent mean ± S.E.M., n = 9–12 (*, P < 0.05 and **, P < 0.01, compared with any other column of the panel). D, SHR were treated for 2 weeks with vehicle (Ctr), apocynin (Apo; 70 mg/kg/day), diclofenac (Dic; 5 mg/kg/day), or a combination of apocynin and diclofenac (D+A). Immunohistochemical analyses of aortic sections were performed using an anti-nitrotyrosine antibody and the avidin/biotinylated enzyme (peroxidase) complex system. The reaction was visualized with 3,3’-diaminobenzidine and counterstained with hematoxylin.
However, NSAID-induced up-regulation of NADPH oxidases and the resulting oxidative stress are probably independent of changes in blood pressure, because they could also be demonstrated in cultured endothelial cells (Fig. 6, A and B).

As a last piece of evidence, the current study demonstrates in healthy volunteers that the nitroglycerin-induced, NO-mediated nitroglycerin-induced vasodilation was clearly attenuated. At least in SHR, coxibs produce less oxidative stress than the nonselective NSAIDs. The enhanced production of reactive oxygen species in response to COX inhibition could be the reason why total cardiovascular risk for nonselective NSAIDs is not higher than that for coxibs.

In conclusion, we provide evidence that COX inhibition by NSAIDs enhances the expression of vascular and cardiac NADPH oxidases and reduces vascular NO bioavailability in SHR and human endothelial cells. This seems to have functional consequences even in man in vivo, because the NO-mediated nitroglycerin-induced vasodilation was clearly attenuated. At least in SHR, coxibs produce less oxidative stress than the nonselective NSAIDs. The enhanced production of reactive oxygen species in response to COX inhibition may be responsible, at least in part, for the adverse cardiovascular effects of NSAIDs observed in clinical studies.

Acknowledgments
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References

Fig. 6. NSAIDs increase Nox2 expression and reduce bioactive NO in human endothelial cells. A, human EA.hy 926 endothelial cells were treated with rofecoxib, celecoxib, or diclofenac (10 μM each) for 24 h. mRNA expression of Nox2 and p22phox was analyzed with quantitative real-time RT-PCR. B, EA.hy 926 cells were pretreated with rofecoxib, celecoxib, or diclofenac (10 μM each) for 24 h, stimulated with 300 μM ATP for 3 min, and the conditioned media were transferred to RFL-6 reporter cells. cGMP content in RFL-6 cells was determined by radiomunnoassay. Columns represent mean ± S.E.M., n = 6–9 (*, P < 0.05 and **, P < 0.01, compared with control).

Fig. 7. NSAIDs impair the dilation of the human brachial artery in response to nitroglycerin. Healthy volunteers were treated for 5 days with either rofecoxib (50 mg once daily, n = 7), celecoxib (200 mg twice daily, n = 10), or diclofenac (75 mg twice daily, n = 10). Sublingual nitroglycerin (0.8 mg) was applied before and in the morning of the last day of a 5-day treatment period (2 h after the last dose). The diameter of the right brachial arterial was measured noninvasively using high-resolution vascular ultrasound. Data are expressed as percentage change of baseline arterial diameter and represent mean ± S.E.M. (*, P < 0.05 and **, P < 0.01, n.s., not significant.)


Address correspondence to: Dr. Ulrich Förstermann, Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany. E-mail: ulrich.forstermann@uni-mainz.de