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Title Page

COX2-selective and non-selective NSAIDs induce oxidative stress by upregulating vascular NADPH oxidases

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Running Title Page

Running title: NSAIDs induce oxidative stress

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ABSTRACT

Cyclooxygenase 2 (COX2)-selective inhibitors (coxibs) and non-selective non-steroidal anti-inflammatory drugs (NSAIDs) are associated with an increase in cardiovascular events. The current study was designed to test the effect of coxibs and non-selective NSAIDs on vascular superoxide and 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 non-selective NSAIDs diclofenac or naproxen, and moderately by rofecoxib or celecoxib in the aorta and heart of SHR. The upregulation 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 (VASP). 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^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 non-selective NSAIDs being even more critical than coxibs in this respect.

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Introduction

Non-steroidal 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 is also expressed in response to physiologic stimuli (see below). Aspirin and other non-selective 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). When compared with placebo, allocation to a coxib is associated with a 42% relative increase in the incidence 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 prostanoid imbalance theory, COX2 inhibition by coxibs would markedly reduce prostacyclin production from endothelial cells without inhibiting COX1 in platelets, and thus, favor thromboxane production 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 non-selective NSAIDs, which inhibit primarily COX1, have been found associated with an increased rate of cardiovascular events (Hippisley-Cox and Coupland, 2005; Kearney et al., 2006; McGettigan and Henry, 2006).

Thus, prostanoid 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 FitzGerald, 2003; Forstermann and Munzel, 2006), and there are some hints that NSAIDs may affect redox status of vascular tissues. For example, rofecoxib has been shown to increase the susceptibility of human LDL and cell membrane lipids to oxidative modification (Walter et al., 2004). Rofecoxib can also promote non-enzymatic 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 function 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 appear 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 preformed 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).

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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 $C_{17}H_{14}O_4S$, 10 mg/kg/d], MSD, Munich, Germany (obtained before the withdrawal in October 2004), celecoxib {4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide, CAS number 169590-42-5, chemical formula $C_{17}H_{14}F_3N_3O_2S$, 30 mg/kg/d}, Pfizer, Karlsruhe, Germany, diclofenac [2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid, CAS number 15307-86-5, chemical formula $C_{14}H_{11}Cl_2NO_2$, 5 mg/kg/d], Sigma, Deisenhofen, Germany or naproxen [(+)-(S)-2-(6-methoxynaphthalen-2-yl)propanoic acid, CAS number 22204-53-1, chemical formula $C_{14}H_{14}O_3$, 50 mg/kg/d], Sigma, mixed into the chow for two weeks. The doses of the NSAIDs were extrapolated from doses applied to humans. COX1 activity was not affected at the doses selected for rofecoxib (Hocherl et al., 2002) and celecoxib (Kitahara et al., 2002). Apocynin [1-(4-hydroxy-3-methoxyphenyl)ethanone, CAS number 498-02-2, chemical formula $C_9H_{10}O_3$, Sigma, 70 mg/kg/d], a selective NADPH oxidase inhibitor, was given in drinking water. The use of animals in this study complies with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the German law on the protection of animals. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Real-Time RT-PCR for mRNA expression of NADPH oxidase subunits and eNOS.

mRNA expression of eNOS, Nox isoforms of NADPH oxidase (Nox1, Nox2 and Nox4) and NADPH oxidase subunit p22phox was analyzed with quantitative Real-Time RT-PCR using an iCyclerTM iQ System (Bio-Rad Laboratories, Munich, Germany), as previously described (Li et al., 2006). Total RNA was isolated from aorta and heart of SHR using RNeasy Fibrous Tissue Kit (Qiagen, Hilden, Germany). 500ng of total RNA was used for Real-Time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen). Gene

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expression of the target genes was normalized to the endogenous control, GAPDH mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.

Measurement of superoxide concentration 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, Mannheim, Germany) and centrifuged at 2000 x g for 5 min at room temperature. The supernatant was removed and centrifuged at 20,000 x g for 20 min at 4°C. Again the supernatant was removed and centrifuged at 100,000 x g 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 PBS 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, Bad Wildbad, Germany) in the presence of 200 µM NADPH (Daiber et al., 2004).

Measurement of aortic superoxide concentration 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 NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM). Then, lucigenin (5 µM)-derived chemiluminescence was detected in a Lumat LB 9507 (Berthold). The photon counts were normalized for the counting time and the dry weight of aortic tissues (Oelze et al., 2006).

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 (HBSS, PAA Laboratories, Cölbe, Germany) containing 100 µM of the luminol derivative L-012 [8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H)dione] (Daiber et al., 2004; Li et al., 2006). L-012-derived chemiluminescence

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was measured using a Microplate Luminometer (Berthold). The photon counts were normalized for the dry weight of aortic tissue.

Measurement of ROS content by dihydroethidium fluorescence.

The oxidative fluorescent dye dihydroethidium (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 fluorescent 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 tetramethylbenzidine 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After blocking, immunoblotting was performed with a mouse monoclonal P-VASP phosphoserine 239 antibody (clone 16C2) (Merck, Darmstadt, Germany). α-actinin was detected using a mouse monoclonal antibody (Sigma) for normalization. The bands were evaluated by densitometry (Oelze et al., 2000).

Detection of nitrotyrosine in aortic sections.

Frozen sections of aorta (10 µm) were incubated with a rabbit polyclonal anti-nitrotyrosine antibody (Chemicon/Millipore, catalog #06-284) at a dilution of 1:100.

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Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as secondary antibody. After addition of avidin:biotinylated enzyme (peroxidise) complex (Vectastain® ABC kit, Vector Laboratories), the reaction was visualized with 3,3'-diaminobenzidine. Finally, the sections were additionally stained with hematoxylin.

Determination of NO synthesis as nitrite in rat plasma.

Nitrite, the oxidation product of NO, was assayed in rat plasma as a measure of NO synthesis. Nitrite was determined by ozone chemiluminescence after chemical reduction to NO using a NOA^{TM} 280 Nitric Oxide Analyzer (Sievers) (Li et al., 2006).

Measurement of aortic cGMP content.

Aorta segments in 3 mm length were equilibrated for 60 min at 37°C in HBSS containing 200 U/ml SOD, 300 µM 3-isobutyl-1-methylxanthine (IBMX), 100 µM L-arginine, with or without 20 µM (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4). Then, 300 nM acetylcholine were added and an incubation for 3 min was performed to stimulate eNOS. Aortic cGMP content was determined by radioimmunoassay.

Reporter cell assay for determination of bioactive NO in human endothelial cells.

Human EA.hy 926 endothelial cells, derived from human umbilical vein endothelial cells, were treated with either rofecoxib, celecoxib, or diclofenac (10 µM each) for 24 hours. NO production was bioassayed with RFL-6 rat lung fibroblasts used as reporter cells (Li and Forstermann, 2000). Briefly, after the 24h pre-treatment, EA.hy 926 cells were incubated for 3 min with the respective NSAID (10 µM) and 300 µM ATP. Then, the conditioned media were transferred onto the RFL-6 cells. On the RFL-6 cells, another 3-min incubation was performed. The cGMP content of the RFL-6 samples was determined by radioimmunoassay as described (Li and Forstermann, 2000).

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Assessment of nitroglycerin-induced dilatation of the right brachial artery in healthy human volunteers.

Ten healthy volunteers (for baseline clinical characteristics see Table 1) were randomly assigned to 5-day treatments with either rofecoxib (50 mg once daily), celecoxib (200 mg twice daily), diclofenac (75 mg twice daily) or placebo. Volunteers received all three NSAIDs, but only seven of the subjects received rofecoxib due to the restricted availability of the drug. All volunteers were from the scientific laboratory personnel and all gave informed consent to participate in the study. Nitroglycerin-induced dilatation was assessed before and in the morning of the last day of a 5-day treatment period (2 h after the last NSAID dose). Brachial arterial diameter was measured non-invasively (Warnholtz et al., 2005) immediately before and 4 min after sublingual administration of nitroglycerin (0.8 mg, Nitrolingual®, Pohl-Boskamp, Hohenlockstedt, Germany). Two-dimensional baseline images of brachial artery diameter were obtained with an ATL HDI5000 ultrasound system (Philips, Da Best, The Netherlands). Drug free periods of 9 days were allowed between the different NSAID treatments.

Statistics.

Changes of brachial artery diameter in response to nitroglycerin in control and NSAID-treated individuals were compared for statistical differences using Student's t test for paired values. Because the rofecoxib group did not show Gaussian distribution Wilcoxon's signed rank test was used. For all other comparisons of different means analyses of variance (ANOVA) followed by Fisher's protected least-significant-difference test were performed.

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Results

NSAIDs enhance the expression of NADPH oxidases in aorta and heart of SHR.

Treatment of SHR with rofecoxib (10 mg/kg/d), celecoxib (30 mg/kg/d), diclofenac (5 mg/kg/d) or naproxen (50 mg/kg/d) for 2 weeks resulted in enhanced mRNA expression of NADPH oxidases in 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 non-selective 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 aorta from treated SHR rats. 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 in NSAID-treated SHR (Fig. 2E).

Treatment with apocynin, a NADPH oxidase inhibitor, prevented diclofenac-induced ROS content, both in the aorta (Figs. 3A and C) and the heart (Figs. 3B and D).

NSAIDs decrease the level of bioactive NO, but upregulate eNOS expression.

Treatment with NSAIDs decreased the plasma levels of nitrite, the oxidation product of NO (Fig. 4A). eNOS expression, however, was not reduced, but was even upregulated by diclofenac or naproxen (Fig. 4B). In the aorta, VASP phosphorylation was decreased by treatment with NSAIDs (Figs. 4C 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.

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The increased superoxide concentration in diclofenac-treated animals was reduced by NOS inhibitor L-NAME, as determined with L-012- or lucigenin chemiluminescence (Figs. 5A and B). Aortas from diclofenac-treated SHR showed a reduced cGMP content, which could be reversed by NOS cofactor BH_4 (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, 24h, Fig. 6A).

When RFL-6 reporter cells were treated with conditioned media from non-stimulated EA.hy 926 endothelial cells, a basal cGMP level of 2.2±0.1 pmol/10^6 cells was measured. The cGMP level increased to 33.7±1.5 pmol/10^6 cells, when endothelial cells were stimulated with 300 µM ATP. 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/10^6 cells (not shown). Pre-treatment of EA.hy 926 cells for 24h with rofecoxib or diclofenac significantly reduced cGMP levels in the RFL-6 reporter cell assay; a 24h-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 non-invasively using high-resolution vascular ultrasound. All three NSAIDs impaired nitroglycerin-induced dilatation significantly (Fig. 7).

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Discussion

The present study provides evidence in favor of a novel theory explaining the adverse cardiovascular effects of coxibs and non-selective NSAIDs. These data demonstrate that both COX2-selective coxibs (rofecoxib and celecoxib) and, to an even greater extent, non-selective NSAIDs (diclofenac and naproxen) induce oxidative stress in the cardiovascular system of spontaneously hypertensive rats. This is indicated by enhanced vascular superoxide content (Figs. 2A and 2C), 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) upregulated eNOS mRNA expression. The increased expression of eNOS mRNA is likely to be a consequence of an increased production of the reactive oxygen species H_2O_2. H_2O_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 upregulation of eNOS expression (Fig. 4B).

An involvement of the inducible NO synthase (iNOS) in the NSAID-induced reduction in plasma nitrite appears 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 likely reflect NO production from eNOS.

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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 (Forstermann and Munzel, 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 upregulated, 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 (Forstermann and Munzel, 2006).

Indeed, the increased superoxide content in diclofenac-treated SHR could be reduced by the NOS inhibitor L-NAME (Figs. 5A and B), indicating that eNOS was in a dysfunctional state under these conditions. In many cardiovascular disease states associated with eNOS uncoupling, oxidative degradation of the NOS cofactor BH_4 is likely to be the common cause of eNOS dysfunction (Forstermann and Munzel, 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 (Forstermann and Munzel, 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 (Figs. 4C and D) in the aorta. This indicates a functional impairment of the NO/cGMP/cGK pathway (most probably caused by the diminished formation of bioactive NO). VASP is a validated substrate of cGMP-dependent protein kinase (cGK) and phosphorylated VASP (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 non-selective 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 (Figs. 6A and B). This result is

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interesting in view of clinical trials demonstrating a more favorable cardiovascular risk profile of celecoxib relative to rofecocib (McGettigan and Henry, 2006). In an animal study, celecoxib even improved endothelial function, whereas rofecoxib did not (Hermann et al., 2003). Different intrinsic physico-chemical properties of celecoxib (sulphonamide) 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 non-selective NSAIDs can produce increases in blood pressure in rats (Hocherl et al., 2002; Hermann et al., 2003) and in humans (Mukherjee et al., 2001). However, NSAID-induced upregulation of NADPH oxidase and the resulting oxidative stress are likely to be independent of changes in blood pressure, because they could also be demonstrated in cultured endothelial cells (Figs. 6A and B).

As a last piece of evidence, the current paper demonstrates in healthy volunteers that the nitroglycerin-induced, NO-mediated vasodilation was impaired by NSAIDs (Fig. 7). The reduced relaxation in response to nitroglycerin could be due to several mechanisms: (i) enhanced oxidative stress by NSAIDs (induction of NADPH oxidase and uncoupling of eNOS) can reduce the bioavailability of nitroglycerin-derived NO (Mollnau et al., 2002); (ii) NSAIDs, by inducing oxidative stress, may lower the expression and activity of guanylyl cyclase (Munzel et al., 2005; Gerassimou et al., 2007), and (iii) endogenous prostacyclin can contribute to the relaxing effect of nitroglycerin (Hink et al., 2003). Thus, NSAIDs could also reduce the relaxation in response to nitroglycerin by blocking prostacyclin synthesis.

Because eNOS-derived NO is vasoprotective whereas vascular oxidative stress facilitates vasospasm and promotes atherosclerosis (Forstermann and Munzel, 2006), the data of the current study can (at least in part) provide a potential pathomechanism for the outcome of clinical studies demonstrating that coxibs and non-selective NSAIDs increase cardiovascular events, especially in high-risk patients.

Whereas both coxibs and non-selective COX inhibitors produce oxidative stress, this was more pronounced with the non-selective NSAIDs (Fig. 2). This correlates with a clinical study reporting a similar risk of myocardial infarction for celecoxib and rofecoxib, but a somewhat higher risk for diclofenac (Hippisley-Cox and Coupland, 2005). In other studies,

however, rofecoxib has been found associated with a higher risk for cardiovascular disease than diclofenac (Kearney et al., 2006; McGettigan and Henry, 2006). Thus, oxidative stress is likely to represent one of the mechanisms responsible for adverse cardiovascular effects of NSAIDs, but not the only one. Other contributing mechanisms include effects on prostanoids regulating platelet-vessel wall interactions. Here the non-selective NSAIDs prevent platelet aggregation/adhesion (by inhibiting COX1-dependent thromboxane formation), whereas coxibs reduce preferentially vessel wall-derived prostacyclin (Antman et al., 2005). This could be the reason why total cardiovascular risk for non-selective 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 oxidase 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 non-selective 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.

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Footnotes:

*Both authors contributed equally to this paper.

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Legends for Figures

Fig. 1. NSAIDs enhance the mRNA expression of NADPH oxidases in aorta and heart of spontaneously hypertensive rats (SHR). SHR were treated with rofecoxib (10 mg/kg/d), celecoxib (30 mg/kg/d), diclofenac (5 mg/kg/d) or naproxen (50 mg/kg/d) for 2 weeks and mRNA expression of NADPH oxidases in aorta (panel A) and heart (panel B) was analyzed with quantitative Real-Time RT-PCR. Columns represent mean ± SEM, n = 5-12 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with control).

Fig. 2. NSAIDs induce oxidative stress in SHR. SHR were treated with rofecoxib (Rof, 10 mg/kg/d), celecoxib (Cel, 30 mg/kg/d), diclofenac (Dic, 5 mg/kg/d) or naproxen (Nap, 50 mg/kg/d) for 2 weeks. Reactive oxygen species (ROS) in SHR aorta (panel A) or heart (panel B) were determined with fluorescent microscopy using dihydroethidium. Panel C: ROS content in aortic rings was measured by L-012-derived chemiluminescence. Panel D: Superoxide concentration in heart membrane fractions studied with Lucigenin (5 μM) chemiluminescence. Panel E: plasma total peroxides measured photometrically. Columns represent mean ± SEM, n = 9-24 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with control, Ctr).

Fig. 3. Apocynin prevents diclofenac-induced oxidative stress in SHR. SHR were treated for 2 weeks with vehicle (Ctr), the NADPH oxidase inhibitor apocynin (Apo, 70 mg/kg/d), diclofenac (Dic, 5 mg/kg/d) or a combination of both (D+A). ROS content in SHR aorta (panel A) or heart (panel B) was detected with dihydroethidium fluorescent microscopy, or in aortic rings (panel C) or heart membrane fractions (panel D) determined by lucigenin chemiluminescence. Columns represent mean ± SEM, n = 9-12 (* $P<0.05$, compared with any other column within the panel).

Fig. 4. NSAIDs decrease NO bioactivity, increase eNOS expression and reduce VASP phosphorylation. SHR were treated with rofecoxib (10 mg/kg/d), celecoxib (30

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mg/kg/d), diclofenac (5 mg/kg/d) or naproxen (50 mg/kg/d) for 2 weeks. Plasma nitrite was determined by ozone chemiluminescence (NO-Analyzer, panel A). eNOS mRNA expression in aorta was analyzed with quantitative Real-Time RT-PCR (panel B). VASP phosphorylation (panels C and D) 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 ± SEM, n = 3-12 (* P<0.05, ** P<0.01, *** P<0.001, compared with control).

Fig. 5. Diclofenac induces eNOS uncoupling in SHR aortas and leads to tyrosine nitration (indicative of peroxynitrite formation). SHR were treated for 2 weeks with vehicle (Ctr) or diclofenac (Dic, 5 mg/kg/d, panels A-C). Superoxide content in aortic rings was determined with L-012- (panel A) or lucigenin (Luc.) chemiluminescence (panel B) in the absence or presence of the NOS inhibitor L-NAME (L-N, 1 mM). Panel C: aortic rings were pretreated with (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4), and then stimulated with 300 nM acetylcholine. cGMP content in the aortic tissues was determined with radioimmunoassay. Columns represent mean ± SEM, n = 9-12 (* P<0.05, ** P<0.01, compared with any other column of the panel). Panel D: SHR were treated for 2 weeks with vehicle (Ctr), apocynin (Apo, 70 mg/kg/d), diclofenac (Dic, 5 mg/kg/d) 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.

Fig. 6. NSAIDs increase Nox2 expression and reduce bioactive NO in human endothelial cells. Panel A: Human EA.hy 926 endothelial cells were treated with rofecoxib, celecoxib, or diclofenac (10 μM each) for 24 hours. mRNA expression of Nox2 and p22phox was analyzed with quantitative Real-Time RT-PCR. Panel B: EA.hy 926 cells were pre-treated with rofecoxib, celecoxib, or diclofenac (10 μM each) for 24 hours, stimulated with 300 μM ATP for 3 min and the conditioned media transferred to RFL-6 reporter cells. cGMP

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content in RFL-6 cells was determined by radioimmunoassay. Columns represent mean ± SEM, n=6-9 (*$P < 0.05$, ** $P < 0.01$ compared with control).

Fig. 7. NSAIDs impair the dilatation 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 hours after the last dose). The diameter of the right brachial arterial was measured non-invasively using high-resolution vascular ultrasound. Data are expressed as percent change of baseline arterial diameter and represent mean ± SEM (*P<0.05, ** P<0.01).

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Table 1. Laboratory parameters of the ten volunteers receiving rofecoxib (50 mg once daily), celecoxib (200 mg twice daily) or diclofenac (75 mg twice daily), each for five days. The volunteers had the following baseline clinical characteristics: age: 35±3 years, male: 50%, body mass index: 23.0±0.9 kg/m^2, hypertriglyceridemia: 20%, hypertension: 0%, diabetes: 0%, smoker: 10%. All measured laboratory parameters were normal for all volunteers.

	Before placebo	After placebo	Before rofecoxib	After rofecoxib	Before celecoxib	After celecoxib	Before diclofenac	After diclofenac
Total cholesterol (mg/dl)	155.1±8.3	150.3±7.4	158.5±11.4	147.6±10.7	158.6±11.8	154.0±9.2	152.9±11.4	156.5±9.1
LDL cholesterol (mg/dl)	82.2±9.1	84.4±8.8	79.4±10.4	75.1±10.5	85.9±10.0	81.0±8.6	83.1±9.4	84.4±8.6
HDL cholesterol (mg/dl)	54.1±3.8	55.2±3.3	55.5±4.1	55.3±3.6	53.6±3.2	52.7±3.2	53.6±3.2	51.7±2.9
Glucose (mg/dl)	89.9±4.3	89.0±3.0	87.4±3.3	89.3±3.5	90.0±3.3	88.6±3.4	87.8±1.4	88.7±2.3
Brachial arterial diameter (mm)	4.07±0.20	4.09±0.22	4.01±0.22	4.20±0.17	4.12±0.22	4.14±0.25	4.08±0.22	4.07±0.18

Data are expressed as mean ± SEM; n = 7-10; no significant differences were observed for any of the laboratory parameters before and after NSAID treatment.

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Figure 1

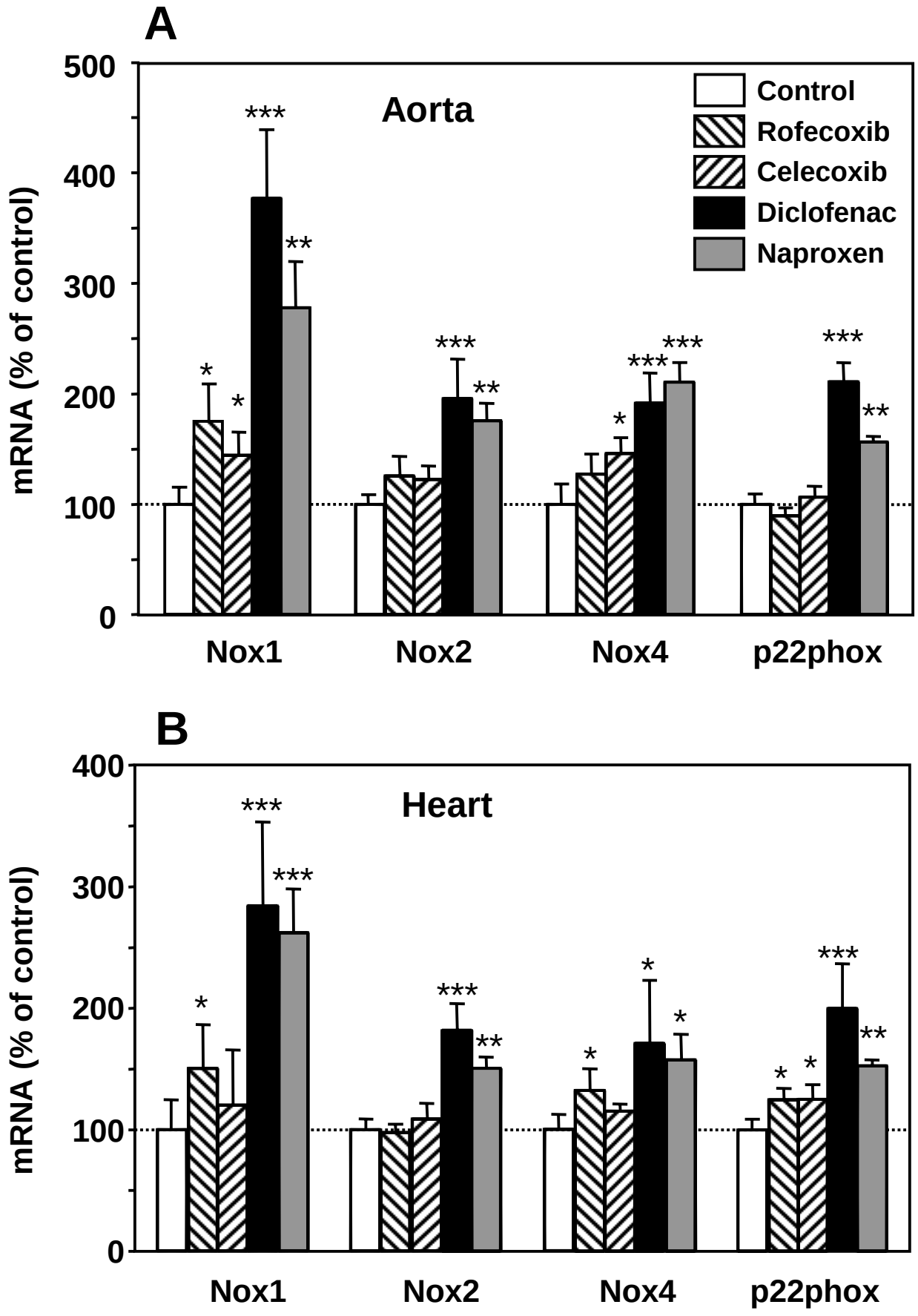

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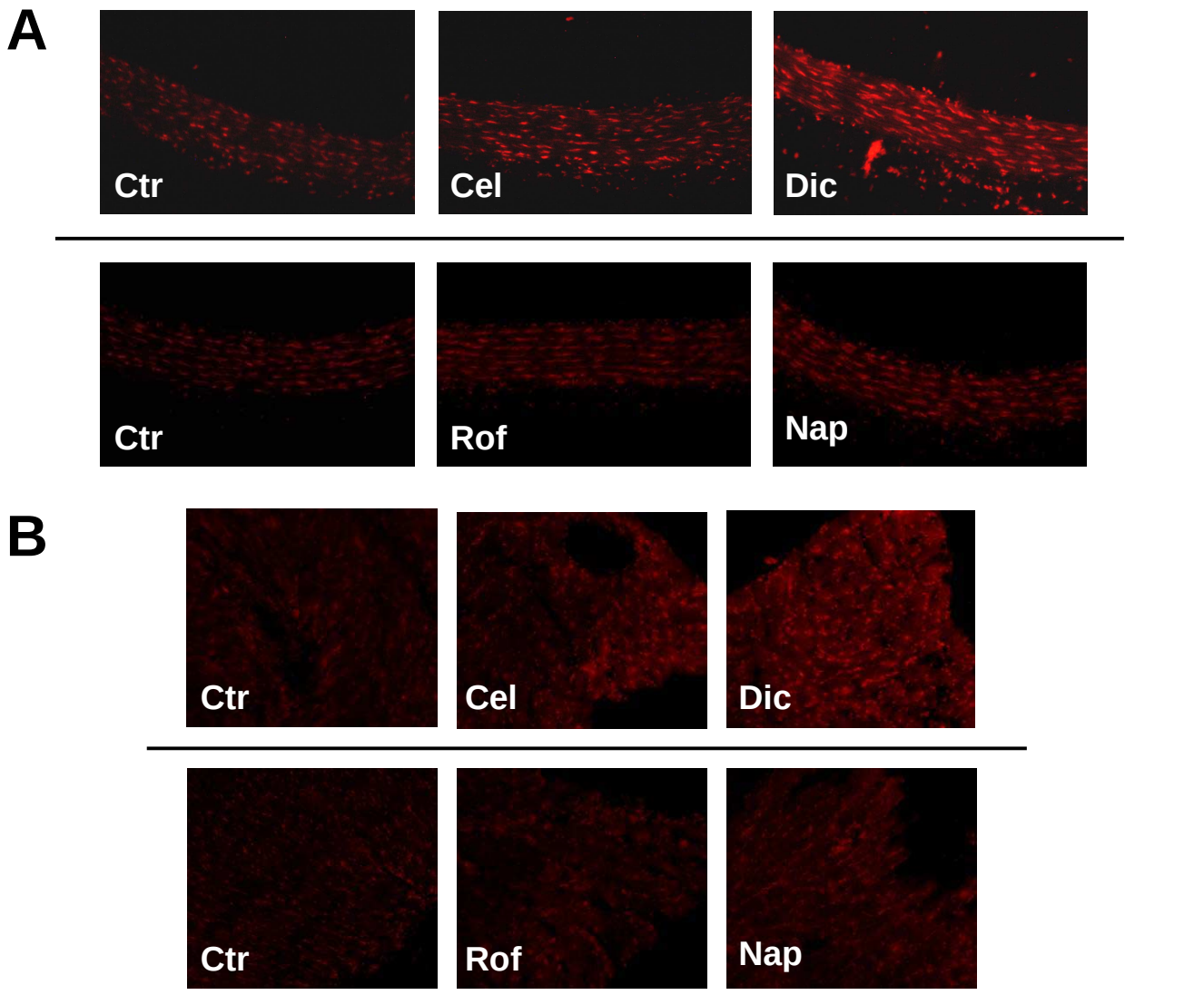
Figure 2



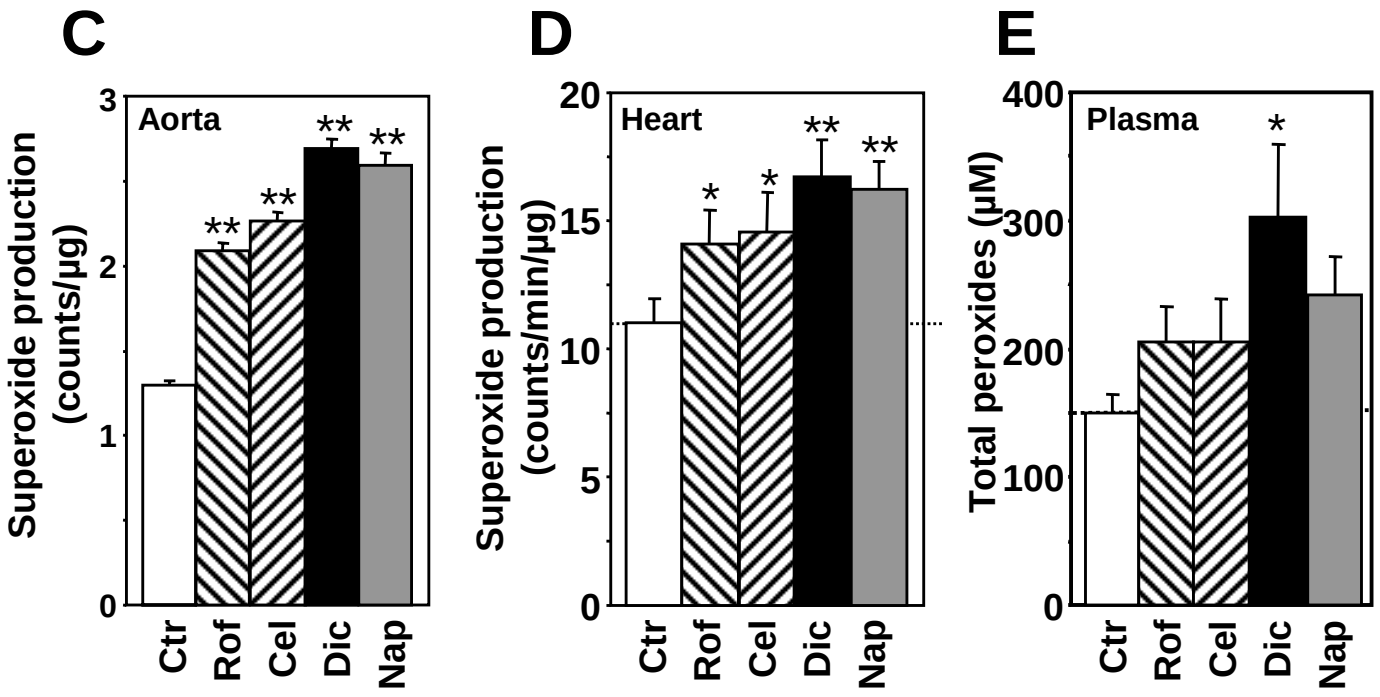


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Figure 3

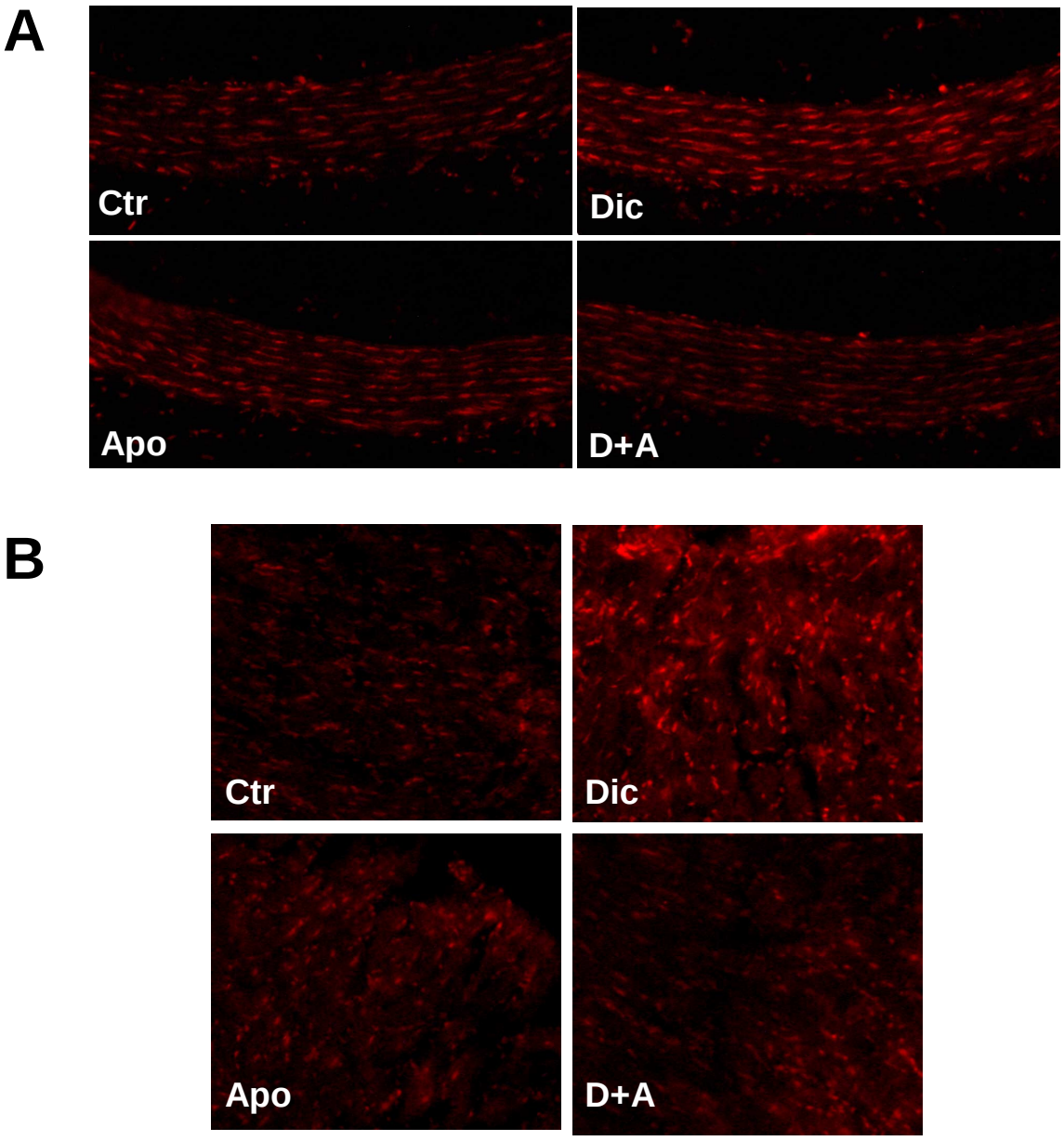

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Figure 4

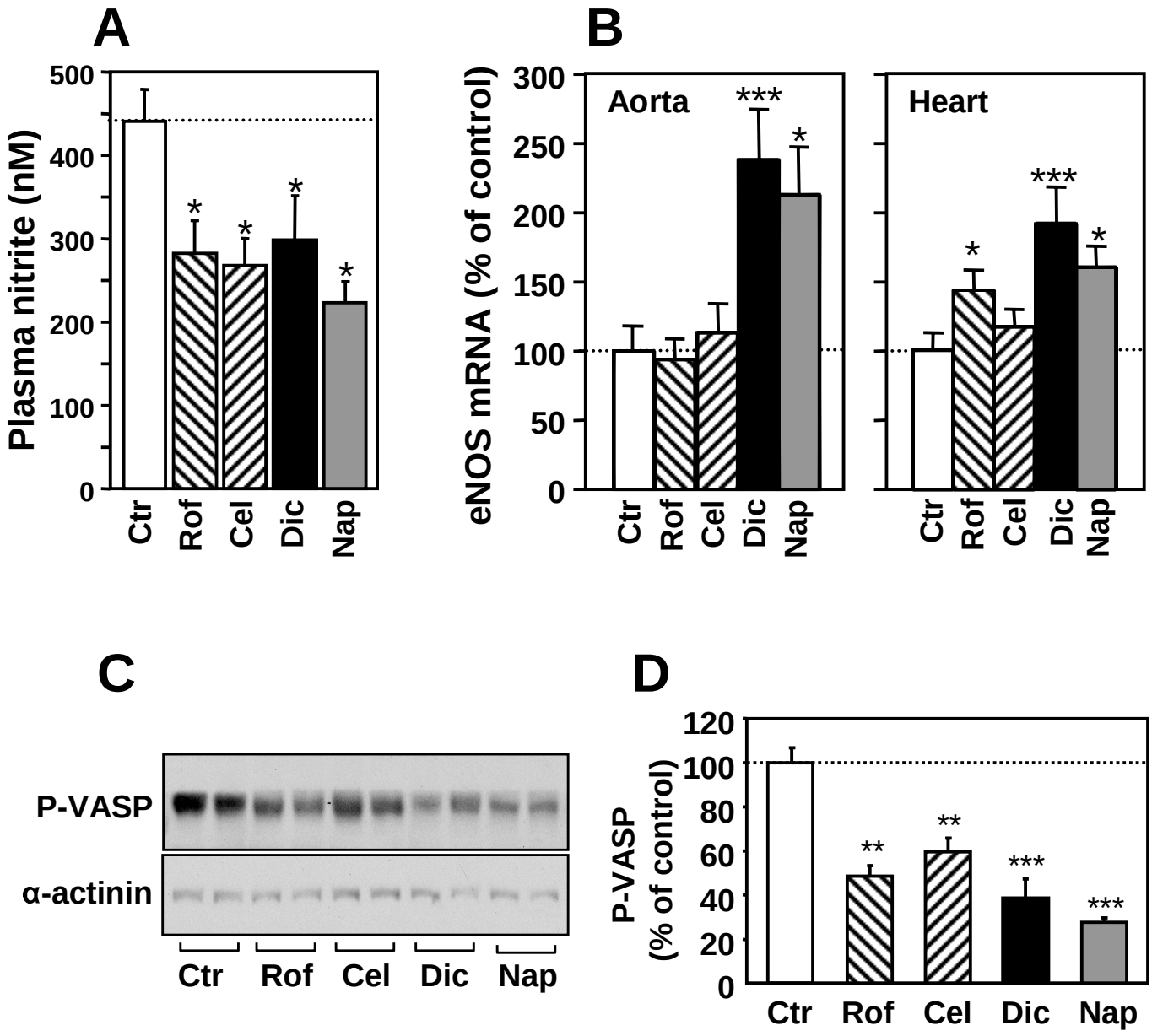

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Figure 5

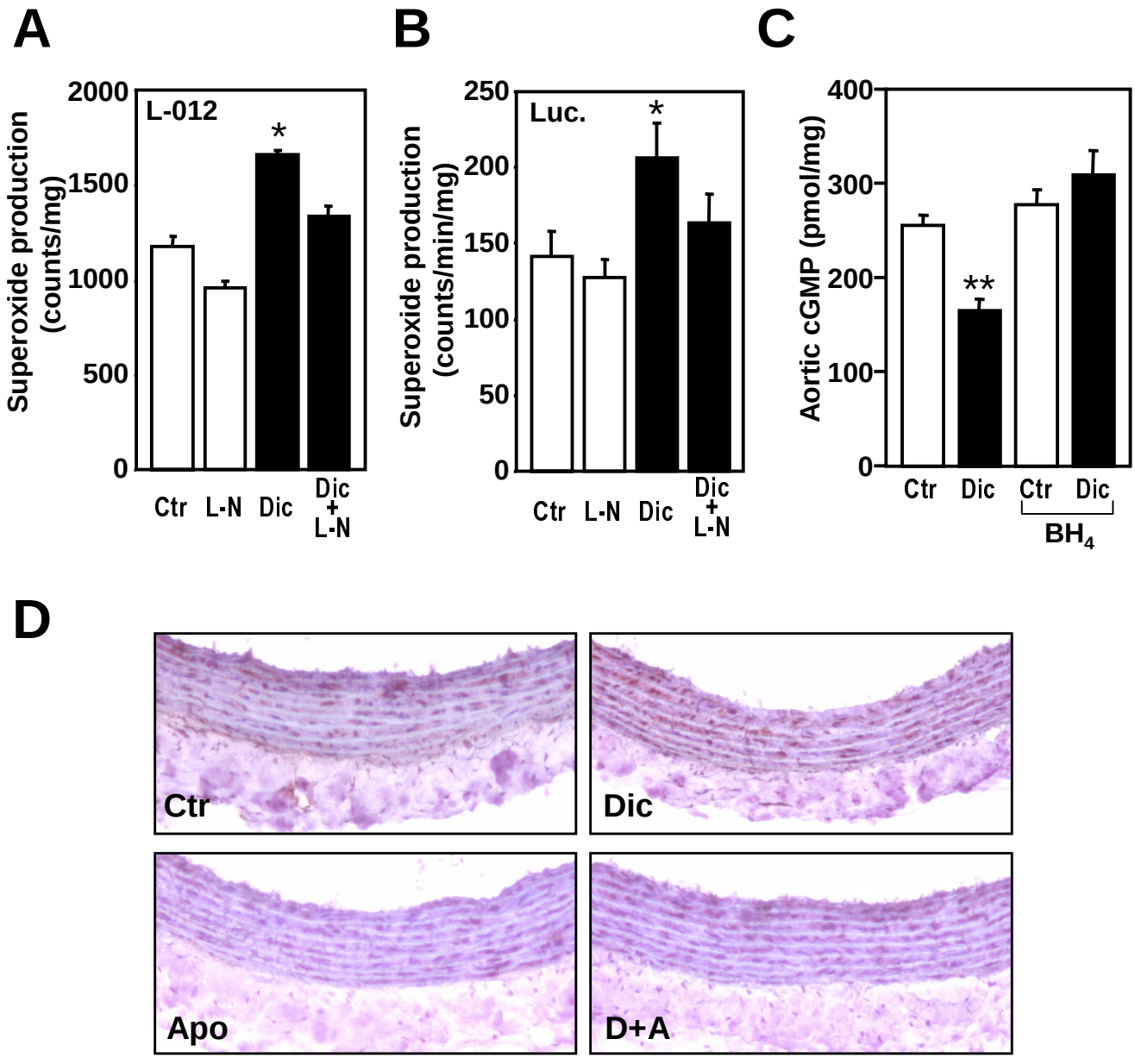

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Figure 6

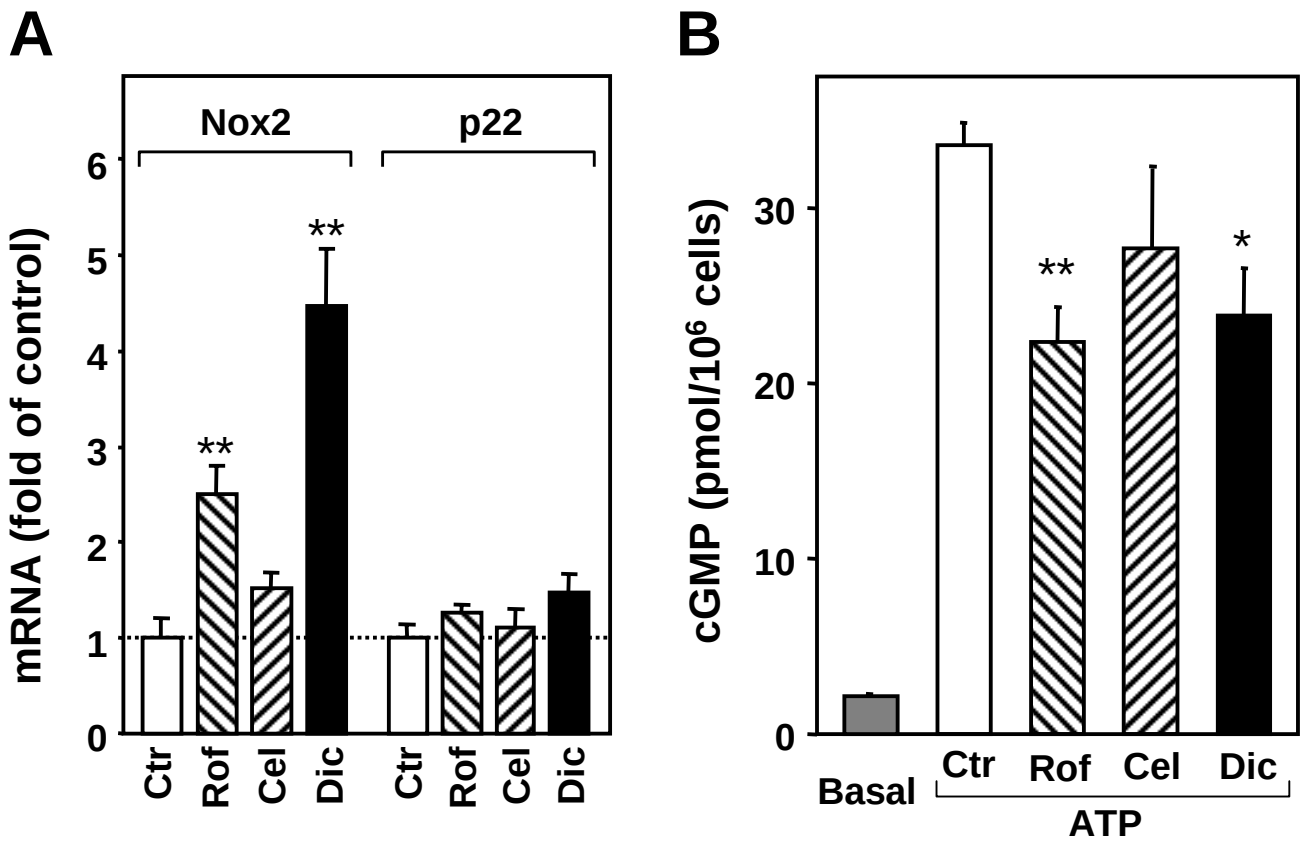

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Figure 7