EFFECT OF NSAID

Endothelial-independent vasorelaxant effect of the non-steroidal anti-inflammatory drugs diclofenac and flufenamic acid on rat isolated aortic vascular rings

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ABSTRACT

Background: Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) that is frequently prescribed as an analgesic agent. Most of the NSAIDs' pharmacological effects are attributed to the inhibition of cyclo-oxygenase (COX) enzymes, but they also have COX-independent actions. Notably, diclofenac has substantial cardiovascular side effects, of which the underlying mechanisms are not fully understood.

Aim: We investigated the effect of diclofenac and the structurally-related COX-inhibiting NSAID flufenamic acid on the contractile activity of aortic vascular rings.

Methods: The contractile force of rat aortic rings was measured using a tension transducer coupled to a PowerLab data-acquisition system. Diclofenac or flufenamic acid was applied on phenylephrine pre-contracted aortic rings. Carbachol was used to induce endothelial-dependent relaxation, whereas the endothelial function was eliminated by denudation of the intimal surface.

Results: Diclofenac induced a dose-dependent relaxation of phenylephrine pre-contracted aortic rings (EC_{50}≈10μM), but had no effect on unstimulated rings. The addition of carbachol to diclofenac, significantly induced further relaxation. Similar results were obtained with flufenamic acid. In endothelium-denuded vessels, either diclofenac or flufenamic acid induced a relaxation of phenylephrine pre-contracted aortic rings, and carbachol had no additional effect.

Conclusion: Diclofenac and flufenamic acid induced aortic vascular relaxation through an endothelial-independent mechanism, but the involvement of COX inhibition cannot be ruled out. The results shed novel insights into the potential therapeutic or adverse effects of diclofenac on vascular function.

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INTRODUCTION

Diclofenac [2-(2, 6-dichloranilino) phenyl acetic acid] is a non-steroidal anti-inflammatory drug (NSAID) that is widely used as an analgesic agent.[1] Generally, diclofenac and other structurally-related non-selective NSAIDs used in experimental settings such as flufenamic acid exert their pharmacological effects through the inhibition of cyclo-oxygenase (COX) enzymes such as COX 1 and COX 2, which catalyse the synthesis of bioactive prostanoids.[2,3] The prostanoids include thromboxane A2 and prostaglandins, and mediate various physiological effects at target sites such as the heart, blood vessels, platelets, and kidneys. However, diclofenac also has COX-independent effects such as the modulation of ion channel expression and activity,[4-7] of which the (patho)physiological role is not fully understood.

The known adverse effects of NSAIDs include gastrointestinal mucosal erosion, renal impairment, platelet dysfunction,[2] and the increased risk of cardiovascular disease.[8,9] In particular, a high cardiovascular risk has been associated with COX-2 selective NSAIDs,[10] which in some instances, has led to the withdrawal of drugs such as rofecoxib from the market.[11] Notably, although diclofenac inhibits both COX 1 and COX 2, it is also fairly COX 2 selective,[12] but the implications of that type of COX selectivity profile on the cardiovascular system are still not fully known. Furthermore, diclofenac is reported to have a higher cardiovascular risk score and greater cardiotoxicity compared to other non-selective NSAIDs such as ibuprofen and naproxen.[13-15]

In the vascular system, there is uncertainty regarding the nature of cardiovascular risk associated with the clinical use of diclofenac,[16,17] in part, because several clinical trials evaluate

the vascular effects of multiple NSAIDs at the same time, making it difficult to attribute any of the overall effects to a particular drug.[18] Diclofenac has been implicated in the coronary artery diseases,[1,19] and its accidental intra-arterial injection has been reported to induce severe vasoconstriction.[20] The drug also impairs the development of the vascular structural components in zebrafish,[21] but clinically does not seem to contribute to cardiovascular events related to changes in blood pressure.[22] Therefore, the fundamental vascular effects of diclofenac still remain insufficiently understood to account for either its therapeutic or adverse effects. In the present study, we evaluated the effect of diclofenac or the structurally-related NSAID flufenamic acid on the aortic vascular tone and explored the possible underlying mechanisms.

METHODS

Drugs and chemicals

Analytical-grade chemicals and drugs were purchased from Sigma-Merck (South Africa), unless stated otherwise. Each of the test drugs (diclofenac, flufenamic acid, or carbachol) was dissolved in DMSO (final dilution <0.1% v/v), whereas phenylephrine was dissolved in water.

Animals and tissue harvesting

Seventeen adult male Wistar rats (250 - 300g) were used in this study. The study was approved by the Faculty of Health Sciences Animal Research Ethics Committee of the University of Cape Town (AEC Protocol 014-014). All procedures on animals were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 2011). The rats were housed under standardised conditions (12 hour light / dark cycle and temperature of 23°C) and had unlimited access to rat chow and water.

Rat tissues were harvested as previously described.[23,24] Briefly, rats were injected intraperitoneally (i.p.) with heparin (500IU/kg) and anaesthetised with sodium pentobarbital (70mg/kg, i.p.; Vetserv, South Africa). Upon the loss of pedal withdrawal reflexes, the heart and aorta were excised through a thoracotomy incision and placed in cold (4°C), oxygenated (95% O_2 and 5% CO_2), and filtered (7-µm pore Whatman filter paper, Sigma-Merck, South Africa) modified Krebs Henseleit solution containing (in mmol/l): 118.5 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 1.2 KH_2PO_4 and 11 glucose (pH 7.4). The descending thoracic aorta was carefully dissected from connective tissues and cut transversely into cylindrical aortic rings (each approximately 4mm long). About 4 aortic rings were obtained from each rat, and each ring was used for a different type of experiment as described below.

Vascular reactivity measurements

Each aortic ring was threaded with 2 stainless steel metal hooks through the lumen (taking care not to damage the endothelial lining) and hanged horizontally in a 30ml water-jacketed, temperature-regulated (37°C) organ bath containing Krebs Henseleit solution (Figure 1A). The Krebs Henseleit solution in the organ bath was bubbled with carbogen (95% O_2 and 5% CO_2) and was renewed at a rate 20ml per hour. The bottom hook was connected to a holder positioned at the base of the organ bath using a surgical string, whereas the top hook was connected, by way of another string, to a tension transducer (MLT050/ST, ADInstruments, Australia), which was coupled to a PowerLab (8/30) data-acquisition system (ADInstruments, Australia). The tension transducer was mounted on an adjustable micro-positioner (MLA41, ADInstruments, Australia). The set up enabled the contractile activity of the aortic ring to be transmitted through the metal hooks and strings to the tension transducer (Figure 1A).

The aortic ring was stabilised for 30 minutes at a pre-tension of 1.5g, achieved by adjusting the micro-positioner. After stabilisation, the transducer tension was calibrated as the zero point (0g), and the aortic ring was contracted using phenylephrine (3µM or 10µM). The test drug doses were added cumulatively as follows: Carbachol (3µM and 100µM), diclofenac (3µM, 10µM, 30µM, and 100µM), and flufenamic acid (3µM, 10µM, 30µM, and 100µM). The test drugs were applied either in the absence of phenylephrine (baseline condition) or during the application of phenylephrine. To evaluate endothelium-independent effects, the aortic ring endothelium was denuded by gently rubbing the vascular intimal surface with the tip of forceps. Data were recorded online via the PowerLab (8/30) data-acquisition system and analysed using the LabChart Pro 7 software (ADInstruments, Australia). Data points of the diclofenac dose-response curve were fitted with a Boltzmann equation using the Origin 6.1 software (OriginLab Corporation, USA) to obtain the effective concentration that produces 50% of maximal activity (EC_{50}).

Data analysis

Data are expressed as mean and standard deviation (SD) or as box plots, and n indicates the number of replicates. Statistical analysis was conducted using the Statistica (Version 13) programme (TIBCO.com). A Shapiro-Wilk test for normality was used to test the distribution of variables. For parametric data, a paired t-test was used to compare measurements before and after drug application, whereas a Wilcoxon test was used for non-parametric data. Parameters measured in each aortic ring under different conditions were compared using repeated-

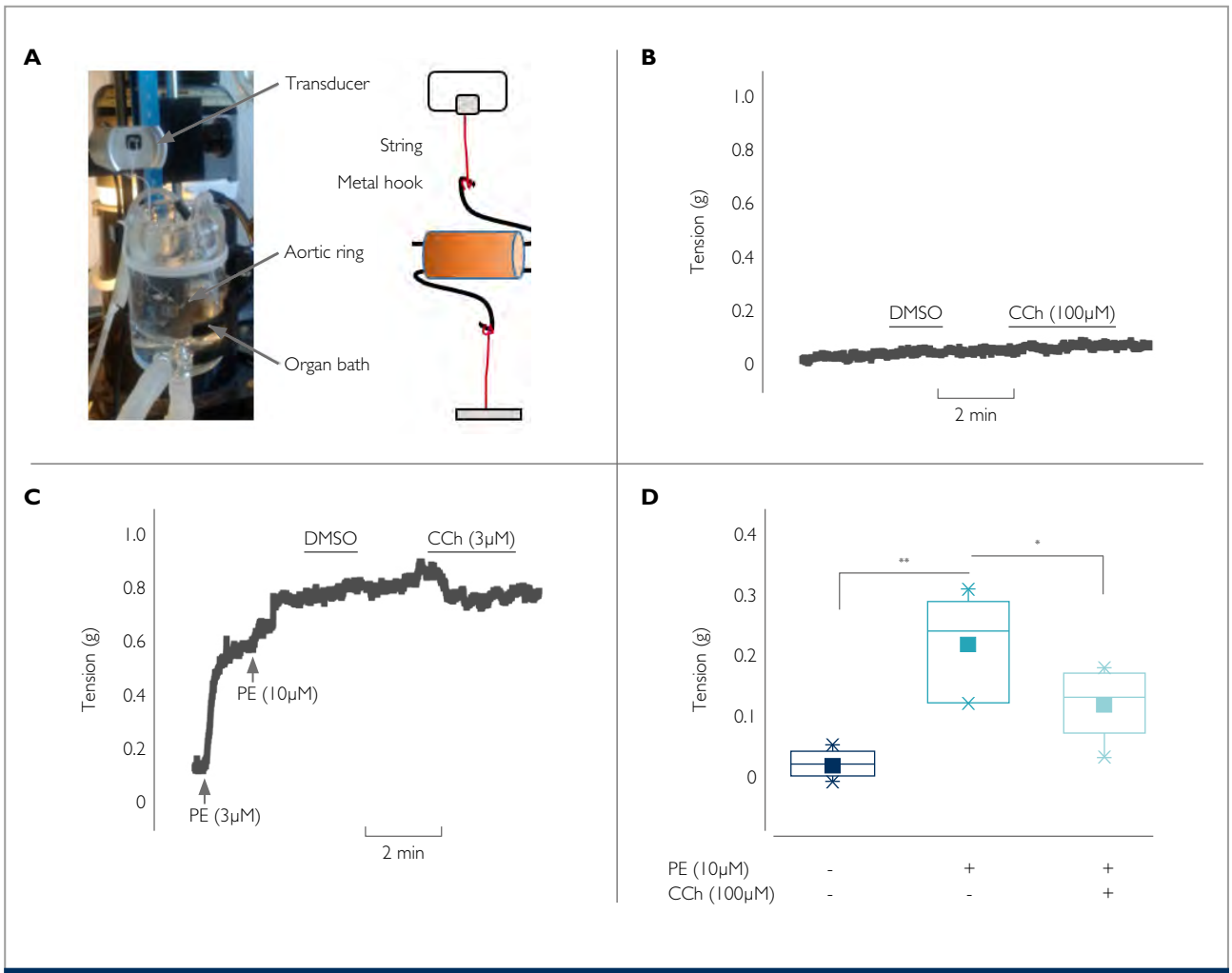

FIGURE 1: Experimental set up and effects of vasoactive drugs.
A: Image of an organ bath and force transducer (left), and schematic of the aortic ring mounting apparatus (right). B and C: Representative tracings of the effects of the vehicle dimethyl sulfoxide (DMSO; 0.1% v/v) and carbachol (CCh) on an unstimulated vessel (B) or on a phenylephrine (PE) pre-contracted vessel (C). D: Quantitative analysis of drug effects on the vascular contractile force (n=6 per group). + or - depicts the presence (+) or absence (-) of a drug. Data are presented as box plot and the mean (filled square). $^{*}p<0.05$; $^{**}p<0.01$.

measures analysis of variance (ANOVA). A 2-tailed p-value <0.05 was considered statistically significant.

RESULTS

Aortic vascular tone and sensitivity to phenylephrine and carbachol

The baseline tension in stabilised aortic vascular rings was relatively steady over time and was altered neither by the vehicle dimethyl sulfoxide (DMSO, 0.1% v/v) nor by 100µM carbachol (Figure 1B). The application of phenylephrine induced an increase in the aortic vascular ring tension with time, until the tension reached a steady-state level (Figure 1C). Carbachol, but not the vehicle DMSO, decreased the tension in phenylephrine pre contracted vascular rings (Figure 1C) by a clinically significant effect size of approximately 50% from 0.21 ± 0.08g [mean (SD)] to 0.11 ± 0.05g [mean (SD)] ($p<0.05$ vs. phenylephrine alone; Figure 1D).

Diclofenac-induced vascular relaxation

In unstimulated aortic vascular rings, the application of diclofenac, on its own, had no effect on the steady-state baseline tension (Figure 2A). However, in phenylephrine pre-contracted aortic rings, diclofenac induced a dose-dependent vascular relaxation, with an effective concentration that produces 50% of maximal activity (EC_{50}) of approximately 10µM (Figure 2B and 2C). The addition of carbachol to the previously applied diclofenac on phenylephrine pre-contracted aortic vascular

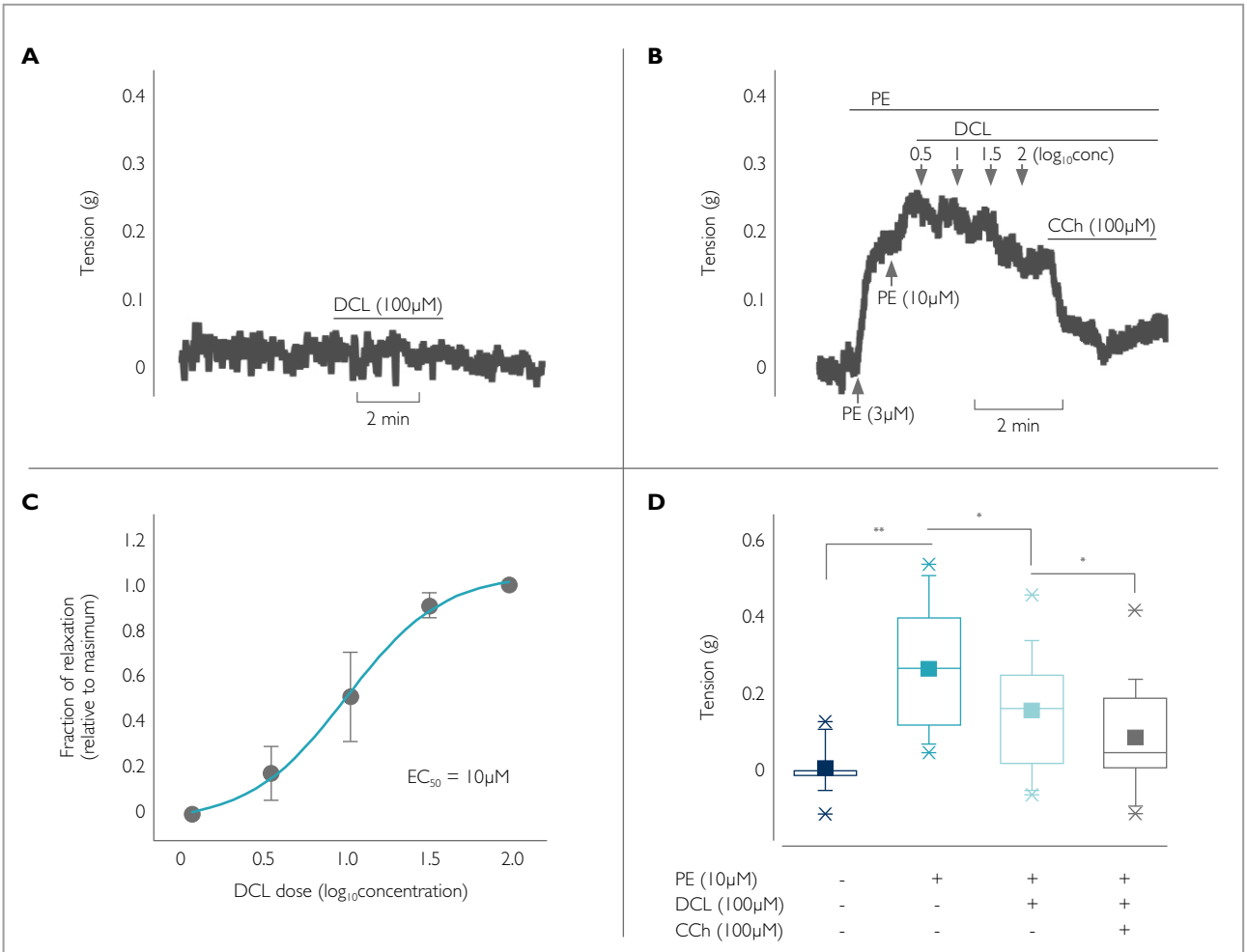

FIGURE 2: Diclofenac-induced vascular relaxation.
A and B: Representative tracings of the effect of diclofenac (DCL) on an unstimulated vessel (A) or on a phenylephrine (PE) pre-contracted vessel (B). The DCL dose is shown as log10 of the concentration. CCh, carbachol. C: The dose-response curve of DCL, with data fitted using a Boltzmann equation (n=12 aortic rings per each data point). Data are presented as mean (SD). EC50, effective concentration that produces 50% of maximal activity. D: Quantitative analysis of drug effects on the vascular contractile force (n=17 per group). + or - depicts the presence (+) or absence (-) of a drug. Data are presented as box plot and the mean (filled square). *p<0.05; **p<0.01.

rings caused a further decrease in tension, beyond the initial effect of diclofenac alone (Figure 2B). As such, the tension after the addition of carbachol to diclofenac was significantly lower than that before carbachol (p<0.05 for before vs. after carbachol; Figure 2D).

Effect of flufenamic acid on the aortic vascular ring tension

To evaluate whether a vasorelaxant effect similar to that of diclofenac could also be produced by another COX-inhibiting NSAID, a non-selective COX inhibitor flufenamic acid was tested. Structurally, both flufenamic acid and diclofenac contain a core phenyl-amino-phenyl ring (Figure 3A). Flufenamic acid had no effect on the baseline tension in unstimulated vessels (Figure 3B), but decreased the tension in phenylephrine pre contracted aortic vascular rings (p<0.05 vs. phenylephrine alone; Figure 3C and 3D). The addition of carbachol to the previously applied flufenamic acid on phenylephrine pre-contracted aortic vascular rings caused a further decrease in tension that was significantly lower than that before the carbachol application (p<0.05 for before vs. after carbachol; Figure 3D).

Effect of endothelial denudation on diclofenac-induced vascular response

To evaluate the contribution of endothelium-dependent activity to the effect of diclofenac, endothelium-denuded vessels were used. The application of phenylephrine induced an increase in the aortic vascular ring tension (p<0.05 vs. baseline; Figure 4A), but as would be expected in endothelium-denuded vessels,

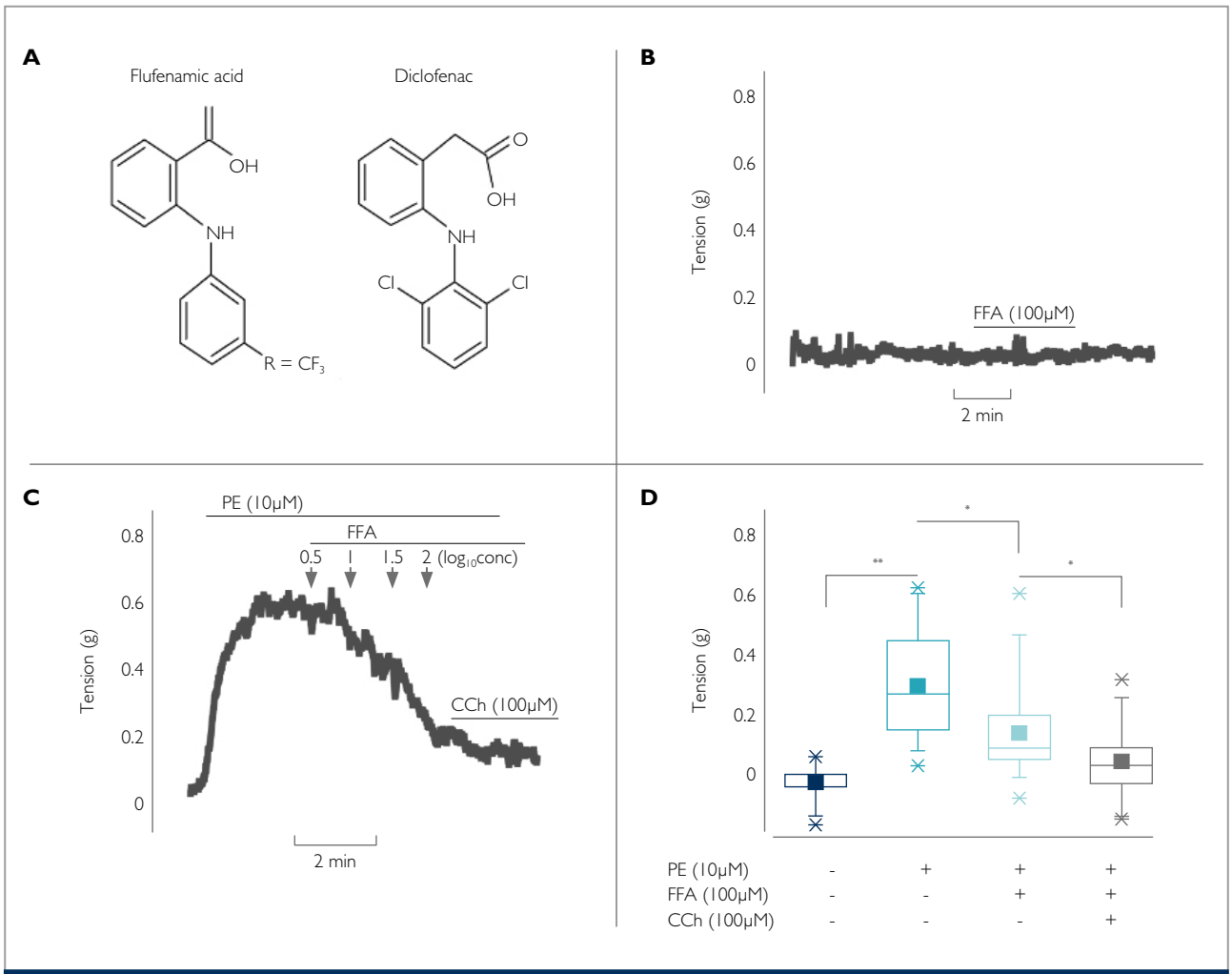

FIGURE 3: Effect of flufenamic acid on aortic vascular reactivity.
A: Chemical structures of flufenamic acid (FFA) and diclofenac (DCL). B and C: Representative tracings of the effects of FFA on an unstimulated vessel (B) and on a phenylephrine (PE) pre-contracted vessel (C). The FFA dose is shown as $\log_{10}$ of the concentration. CCh, carbachol. D: Quantitative analysis of drug effects on the vascular contractile force (n=17 per group). + or - depicts the presence (+) or absence (-) of a drug. Data are presented as box plot and the mean (filled square). $^{*}p<0.05$; $^{**}p<0.01$.

carbachol had no effect on the phenylephrine pre contracted aortic vascular ring (Figure 4A). Diclofenac decreased the vascular tension of the phenylephrine pre contracted, endothelium-denuded aortic vascular rings ($p<0.05$ vs. phenylephrine alone; Figure 4B and 4C). The addition of carbachol to the pre existing diclofenac did not induce further vascular relaxation ($p>0.05$ for before vs. after carbachol; Figure 4B and 4C). Similarly, flufenamic acid decreased the vascular tension in phenylephrine pre contracted endothelium-denuded vessels, whereas the addition of carbachol on top of flufenamic acid had no further effect (Figure 4D).

DISCUSSION

The present study showed a dose-dependent vasorelaxant effect of diclofenac and flufenamic acid on isolated aortic rings as was evidenced by a decrease in phenylephrine-induced contraction. Although the experiments in the present study were performed on isolated vessels, the diclofenac vasorelaxant effect, with an $EC_{50}\approx10\mu M$, occurred at a dose that is clinically relevant, given that the peak plasma level of diclofenac at 2 hours after oral (50mg) dose is 1 µg/ml.[25] Such a dose translates to approximately 3.4µM plasma concentration in an average adult person, which is in the same order of magnitude as the diclofenac EC_{50} observed in the present study. However, as reported in another study on renal vessels, diclofenac had no effect on the contractile force, but instead, decreased the luminal area of phenylephrine-stimulated vessels,[26] probably indicating the tissue-specificity of the NSAID effects. Nevertheless, the vasorelaxant effect of diclofenac observed in the

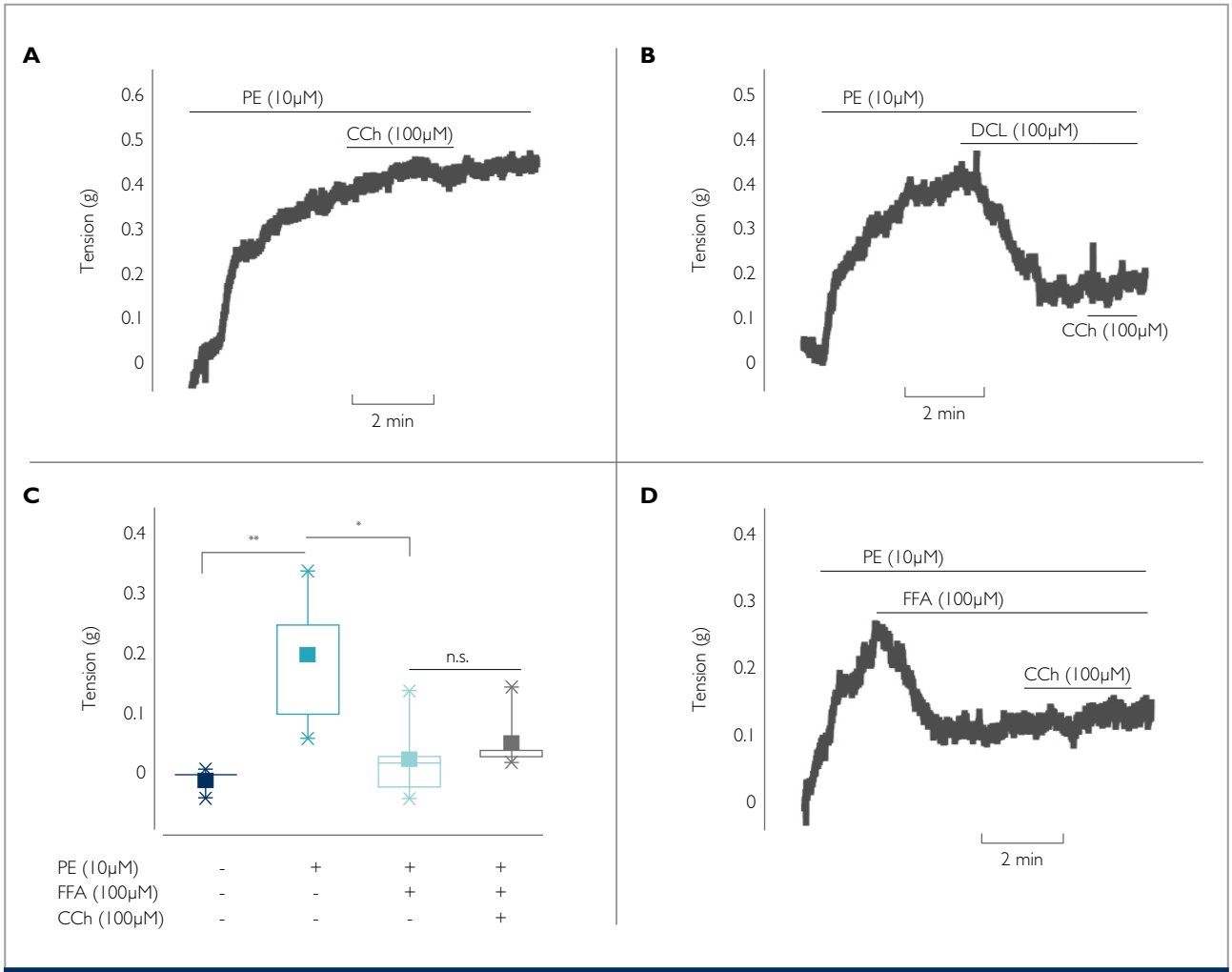

FIGURE 4: Effect of endothelial denudation on the NSAID vascular action.
A and B: Representative tracings of the effects of phenylephrine (PE), carbachol (CCh), and diclofenac (DCL) on the contractile force of endothelium-denuded vessels. C: Quantitative analysis of drug effects on the vascular contractile force (n=5 per group). + or - depicts the presence (+) or absence (-) of a drug. Data are presented as box plot and the mean (filled square). *p<0.05; **p<0.01; n.s., non significant. D: Representative tracing of the effects of phenylephrine (PE) and flufenamic acid (FFA) on the contractile force of an endothelium-denuded vessel (n=4 replicates).

present study is novel in that the generally expected effect of the NSAID inhibition of the constitutively active COX-1 in blood vessels would be to suppress the production of the physiological vasodilatory prostaglandins such as prostacyclin,[2,3] and thereby induce vasoconstriction. As such, this present finding contrasts with the proposed pro-hypertensive effects occurring via COX inhibition in vascular endothelial cells and smooth muscle cells.[12] The reasons behind these opposed vascular effects are not clear and the overall impact of diclofenac treatment on blood pressure has also remained doubtful.[22] However, given the acute application of NSAIDs in the present study, the vasorelaxant effects may reflect short term effects, whereas the pro-hypertensive effects reported in other studies could reflect long-term effects. In addition, since most clinical trial studies have evaluated multiple NSAIDs concurrently,[18] the specific contribution of an individual drug at a given dose may remain unknown. Therefore, the clinical implications of the observed diclofenac effect remain unclear, but depending on the specific tissue involved and the timing of drug application, the vasorelaxant effect could be beneficial through short term improvements in regional blood flow, but could also become detrimental if severe hypotension is induced.

Mechanistically, the vascular effect of diclofenac observed in the present study appears to be independent of the endothelium, since the diclofenac-induced relaxation was still present in endothelial-denuded vessels, whereas, in intact vessels, the carbachol-induced relaxation was still present, despite the

diclofenac effect. A mechanism involving the inhibition of COX by diclofenac could possibly play a role, given that the effect of diclofenac in the present study could be mimicked by another non-selective COX inhibitor flufenamic acid. However, although, like flufenamic acid, diclofenac is considered a non-selective COX inhibitor (i.e., inhibits both COX 1 and COX 2), it also has a unique profile in that it is fairly COX 2 selective.[12] Therefore, although COX inhibition cannot be ruled out as a possible underlying mechanism, it may not fully account for the vasorelaxant effects of diclofenac and flufenamic acid.

Diclofenac and several other NSAIDs also have COX-independent effects such as the modulation of the expression and activity of cardiovascular ion channels,[4,5] which could account for diclofenac and flufenamic acid effects seen in the present study. Both diclofenac and flufenamic acid have stuctural similarities in that they contain a core phenyl-amino-phenyl ring (Figure 3A), a feature that may contribute to unique mode of actions unrelated to COX inhibition. For diclofenac, such COX-independent effects include the blockade the L type $Ca2^{+}$ channel by diclofenac in cardiomyocytes, an effect which, if it were to occur in vascular smooth muscle cells (though not yet known), could produce a vasodilatory effect that is consistent with the findings in the present study. However, there are some key differences between the effects of diclofenac and flufenamic acid in that flufenamic acid (but not diclofenac) induces a non-selective cation current[27] and blocks $Ca2^{+}$-activated chloride currents.[28] So, the role of ion channel modulation in the effects of NSAIDs on blood vessels remains uncertain. As such, there remains a key limitation of the study in that the mechanisms underlying the endothelial-independent vasorelaxant action of diclofenac remain unclear. In addition, since only 2 NSAIDs were tested in the present study, there was a limited scope to evaluate a broad spectrum of COX inhibition.

The present study showed that diclofenac (at a clinically relevant concentration) and flufenamic acid dose-dependently induced the relaxation of aortic vascular rings. The results suggest that the NSAIDs acted via an endothelial-independent mechanism, but a COX-mediated action cannot be ruled out. The findings provide new insights into the potential therapeutic or adverse short-term vascular effects of diclofenac in clinical practice.

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Conflict of interest: none declared.

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