THE EFFECTS OF DICLOFENAC AND IBUPROFEN ON HEART FUNCTION AND OXIDATIVE STRESS MARKERS IN THE ISOLATED RAT HEART

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EFEKTI DIKLOFENAKA I IBUPROFENA NA FUNKCIJU I BIOMARKERE OKSIDATIVNOG STRESA NA IZOLOVANOM SRCU PACOVA

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ABSTRACT

Eicosanoids lead to the promotion of inflammation, cause fever and pain and have many other effects. NSAIDs block the action of cyclooxygenase (COX) during the process of converting arachidonic acid into inflammatory mediators, thus reducing the symptoms of inflammation. Investigations focusing on nonselective COX inhibitors, used in high doses, revealed harmful effects on myocardial function. The aim of our study was to assess the effects of two nonselective NSAIDs, diclofenac and ibuprofen, on cardiodynamic parameters, coronary flow and oxidative stress biomarkers in isolated rat hearts. The hearts of male Wistar albino rats were excised and retrogradely perfused according to the Langendorff technique at gradually increased coronary perfusion pressures (40–120 cm H_2O). The experiments were performed under controlled conditions (Krebs–Henseleit physiological solution). The hearts were perfused with 10 μmol/l diclofenac and 10 μmol/l ibuprofen. The heart function parameters, including the maximum rate of pressure development (dp/dt max), minimum rate of pressure development (dp/dt min), systolic left ventricular pressure (SLVP), diastolic left ventricular pressure (DLVP), mean perfusion pressure (MBP) and heart rate (HR), were continuously registered. Coronary flow (CF) was measured flowmetrically. Oxidative stress markers, including the index of lipid peroxidation measured as TBARS, nitric oxide measured through nitrites (NO_2^-), superoxide anion radical (O_2^-), and hydrogen peroxide (H_2O_2) in the coronary venous effluent, were assessed spectrophotometrically. Our results showed that diclofenac affected cardiodynamic parameters more significantly than did ibuprofen. Furthermore, the present data indicate that both estimated COX inhibitors do not promote the production of reactive oxygen species.

Keywords: *Diclofenac, Ibuprofen, Nonsteroidal anti-inflammatory drugs, Isolated rat heart, Oxidative stress*

SAŽETAK

Eikosanoidi dovode do zapaljenja, uzrokuju groznicu i bol, i imaju mnoge druge efekte na organizam. NSAID onemogućavaju delovanje ciklooksigenaze (COX) u procesu konvertovanja arahidonske kiseline u medijatore zapaljenja, i na taj način smanjuju simptome zapaljenja. Istraživanja koja se bave primenom neselektivnih inhibitora COX, koji se koriste u visokim dozama, pokazala su njihove štetne efekte na funkciju miokarda. Cilj našeg istraživanja je bio da ispita efekte neselektivnih NSAID, diklofenaka i ibuprofena, na kardiodinamske parametre, koronarni protok i biomarkere oksidativnog stresa izolovanog srca pacova. Srca mužijaka Wistar albino pacova su uzimana i retrogradno perfundovana prema Langedorff-ovoj tehnici sa postepenim povećanjem perfuzionog pritiska (40–120 cm H_2O). Eksperimenti su prvo izvođeni u kontrolnim uslovima (primena fiziološkog Krebs–Henseleit-ovog rastvora), nakon čega su srca perfundovana sa: 10 μmol/l dikolfenaka i 10 μmol/l ibuprofena. Parametri srčane funkcije koji su kontinuirano praćeni su: maksimalna stopa razvoja pritiska (dp/dt max), minimalna stopa razvoja pritiska (dp/dt min), sistolni pritisak u levoj komori (SLVP), dijastolni pritisak u levoj komori (DLVP), srednji perfuzioni pritisak (MBP) i frekvenca srčanog rada (HR). Koronarni protok (CF) je registrovan floumetrijski. Markeri oksidativnog stresa: indeks lipidne peroksidacije meren kao TBARS, azot-monoksid utvrđivan preko nitrata (NO_2^-), superoksid anjon radikal (O_2^-), i vodonik peroksid (H_2O_2) su mereni spektrofotometrijski u koronarnom venskom efluentu. Naši rezultati su pokazali da diklofenak ispoljava značajniji uticaj na kardiodinamske parametre u odnosu na ibuprofen. Pored toga, rezultati ove studije su pokazali da oba ispitivana inhibitora COX ne dovode po produkcije reaktivnih vrsta kiseonika.

Ključne reči: *Diklofenak, Ibuprofen, Nesteroidni-antiinflamatorni lekovi, Izolovano srce pacova, Oksidativni stres*

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ABBREVIATIONS

CF - Coronary flow
COX - Cyclooxygenase
CPP - Coronary perfusion pressure
HRPO - Horseradish peroxidase
MBP - Mean blood pressure
NO - Nitric oxide
NADPH - Nicotinamide adenine dinucleotide phosphate
NSAID - Nonsteroidal anti-inflammatory drugs
ROS - Reactive oxygen species
TBARS - Thiobarbituric acid reactive substances
TRIS - Tris(hydroxymethyl)aminomethane

INTRODUCTION

Cyclooxygenase (COX) is an intracellular enzyme that catalyses the conversion of arachidonic acid into prostaglandin H_2, a precursor for the synthesis of prostaglandins, prostacyclin, and thromboxane, also known as eicosanoids. Eicosanoids lead to the promotion of inflammation, cause fever and pain and have many other systemic effects. There are two isoforms of COX: COX-1 and COX-2. COX-1 is the constitutive isoform of COX, and it has clear physiological effects. The inducible isoform, COX-2, is induced by pro-inflammatory stimuli in migratory cells and inflamed tissues (1). It is almost impossible to detect COX-2 in a normal heart, which indicates that the induction of COX-2 is present mostly at the site of inflammation (2).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most common medications in the world for treating a variety of inflammatory disorders, and they are used as analgesics, anti-inflammatory drugs, and antipyretics (3). NSAIDs block the action of COX in the process of converting arachidonic acid into inflammatory mediators, thus reducing symptoms of inflammation (4). NSAIDs are classified on theas nonselective COX inhibitors and specific COX-2 inhibitors depending on the activity of each isoform (4).

Investigations focusing on nonselective COX inhibitors, used in high dosages, revealed harmful effects on myocardial function and increased mortality in patients with previous myocardial infarction (5) and as well as in patients without a prior clinical diagnosis of cardiovascular diseases (6). Recent data regarding cardiovascular risk associated with selective COX-2 inhibitors are controversial (7, 8). Over the last several years, evidence has accumulated showing that oxidative stress plays an important role in the pathogenesis of cardiovascular disease (9, 10). One of possible mechanisms by which COX inhibitors exhibit their side effects on the cardiovascular system is the induction of reactive oxygen species (ROS) (11, 12).

Diclofenac, a nonselective non-steroidal anti-inflammatory drug, has been widely used as an anti-inflammatory, analgesic, and antipyretic drug. Clinical observations have shown that long-term treatment with diclofenac correlates with the onset or aggravation of congestive heart failure, which can cause serious cardiovascular thromboembolic events, such as myocardial infarction and stroke (13). McGettigan et al claimed that diclofenac has the highest cardiovascular risk score of the nonselective NSAIDs (14). This conclusion might be because only diclofenac inhibits L-type Ca^{2+} channels and the Na^+ current in cardiomyocytes (13).

Ibuprofen is a nonselective non-steroidal anti-inflammatory drug that is often used to relieve fever and the symptoms of arthritis. This drug is well-tolerated with infrequent but well-characterised adverse effects, including heart failure (15, 16). A study on isolated guinea pig hearts showed that ibuprofen could induce cardiac arrhythmias due to the inhibition of Na^+ and Ca^{2+} channels and a decrease of the excitation propagation within the heart (17).

Based on the aforementioned discussion, the aim of the present study was to assess the effects of two nonselective NSAIDs, diclofenac and ibuprofen, on cardiodynamic parameters, coronary flow and oxidative stress biomarkers in isolated rat hearts.

MATERIALS AND METHODS

Preparation of isolated hearts

The hearts (n=24; each group 12 rats) were excised from male, eight-week old Wistar albino rats, with a body mass of 180 g to 240 g (obtained from Military Medical Academy, Belgrade, Serbia), and perfused in a Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary). After a short-term ether narcosis, the animals were killed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK). After urgent thoracotomy and rapid heart arrest by superfusion with ice-cold isotonic saline, the hearts were rapidly excised, isolated and retrogradely perfused via the aorta according to Langendorff's technique at gradually increased coronary perfusion pressures (CPP) (40–120 cm H_2O). The composition of the non-recirculation Krebs–Henseleit perfusate was as follows (mmol/1): NaCl 118, KCl 4.7, $CaCl_2$ x $2H_2O$ 2.5, $MgSO_4$ x $7H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95 % O_2 plus 5 % CO_2 and warmed to 37°C (pH 7.4).

Immediately after the establishment of automatic operation, the sensor was inserted (transducer BS4 73-0184, Experimetria Ltd, Budapest, Hungary) through an opening created in the left atrium of the heart and the destroyed mitral valve into the left ventricle for the continuous registration of myocardial function. Another sensor (perfusion pressure sensor, Experimetria Ltd, Hungary) was set at the same height as the end of the heart perfusion cannula to measure mean blood pressure (MBP).

Physiological assay and experimental protocol

After the heart perfusion started, a 30-min period was allowed for stabilisation of the preparation. It was performed at a basal CPP of 60 cmH_2O. To test coronary vascular reactivity, all hearts were challenged with short-term occlusions (5–30 s) and a bolus injection of 5 mmol/1 adenosine (60 µl at a flow rate of 10 ml/min to elicit maximum CF) during the stabilisation period. The hearts were discarded (approximately 25 %) if the flow did not increase by 100 % over the control value (for both tests). After an equilibration period, CPP was lowered to 50 and 40 cmH2O and then gradually increased to 70, 80, 90,100, 110, and 120 cmH2O to establish coronary autoregulation. Properly performed control experiments were included in the study (i.e., the groups of hearts in which the CPP/CF relationship was investigated twice in the absence of any drug for 120 min). It was important to confirm the stability of the preparation and the insubstantial difference between the responses to the first and second runs of changes in perfusion pressure, as described previously (18). After setting up the control experimental protocol (Krebs–Henseleit physiological solution [control group]), the hearts were perfused with 10 µmol/l diclofenac and 10 µmol/l ibuprofen.

In the control and experimental groups, after placing the sensor in the left ventricle, the following myocardial function parameters were continuously registered:

1. Maximum rate of pressure development in the left ventricle (dp/dt max),
2. Minimum rate of pressure development in the left ventricle (dp/dt min),
3. Systolic left ventricular pressure (SLVP),
4. Diastolic left ventricular pressure (DLVP),
5. Mean perfusion pressure (MBP) and
6. Heart rate (HR).

The test period started immediately after the control experiments to avoid time-dependent adverse effects. The flow was considered stable at each value of perfusion pressure when three repeated values of the CF were the same. The CF was measured flowmetrically with the following parameters:

1. Index of lipid peroxidation (measured as TBARS – thiobarbituric acid reactive substances),
2. Superoxide anion radicals (O_2^-),
3. Hydrogen peroxide (H_2O_2) and
4. Nitric oxide (NO).

The experimental protocol was approved by the Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac, Kragujevac, Serbia.

Biochemical assays

TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by measuring TBARS using 1 % thiobarbituric acid in 0.05 NaOH incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. Krebs–Henseleit solution was used as a blank probe (19).

Determination of superoxide anion radical

The level of superoxide anion radical (O_2-) was measured by a nitro blue tetrazolium reaction in TRIS buffer with the coronary venous effluent at 530 nm. Krebs–Henseleit solution was used as a blank probe (20).

Determination of hydrogen peroxide

The measurement of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red by hydrogen peroxide in a reaction catalysed by horseradish peroxidase (HRPO) (21). Two hundred microlitresd of perfusate was precipitated with 800 ml of freshly prepared phenol red solution, and then 10 µl of (1:20) HRPO (made ex tempore) was added. For a blank probe (instead of coronary venous effluent), an adequate volume of Krebs–Henseleit solution was used. The level of H_2O_2 was measured at 610 nm.

Nitrite determination

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. The nitrite level (NO_2-) was measured and used as an index of nitric oxide (NO) production using Griess's reagent. A total of 0.5 ml of perfusate was precipitated with 200 µl of 30 % sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess's reagent, containing 1 % sulphanilamide in 5 % phosphoric acid/0.1 % naphthalene ethylenediamine dihydrochloride, was added and incubated for 10 min in the dark and measured at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (22).

Drugs

Both diclofenac and ibuprofen were obtained from Sigma-Aldrich Co (USA).

Statistical analysis

Values are expressed as the mean ± SE. A paired t test was used in the statistical analyses; p values less than 0.05 were considered to be statistically significant.

RESULTS

Dp/dt max

Diclofenac at a dose of 10 µmol/l induced a decrease in dp/dt max at CPP = 100 cmH_2O and 120 cmH_2O (Figure 1A), whereas ibuprofen at the same dose did not significantly affect this parameter over the entire CPP range (Figure 2A).

Dp/dt min

Similar to dp/dt max, 10 µmol/l diclofenac significantly affected dp/dt min at CPP = 120 cmH_2O (Figure 1B), and ibuprofen did not cause statistically significant changes in this parameter (Figure 2B).

SLVP

Neither diclofenac nor ibuprofen caused statistically significant changes in SLVP at any CPP (Figure 1C, 2C).

DLVP

There were no changes in the DLVP values during the administration of diclofenac or ibuprofen compared to the controls (Figure 1D, 2D) over the entire CPP range.

Mean perfusion pressure

Diclofenac induced statistically significant changes in the MBP at CPP = 120 cmH_2O (Figure 1E), whereas ibuprofen did not cause any changes in the MBP values compared to the control conditions (Figure 2E).

HR

Both diclofenac and ibuprofen induced statistically significant decreases in HR at CPP = 100 cmH_2O and 120 cmH_2O (Figure 1F, 2F) compared with the control conditions.

CF

Similar to HR, both diclofenac and ibuprofen induced statistically significant decreases in HR at CPP = 100 cmH_2O and 120 cmH_2O (Figure 1G, 2G) compared with the control conditions.

Index of lipid peroxidation (measured as TBARS)

The administration of ibuprofen induced a statistically significant increase in TBARS at CPP = 40 cmH_2O and then a statistically significant decrease at CPP = 100 cmH_2O and 120 cmH_2O (Figure 4A). Diclofenac did not cause any changes in the TBARS values over the entire CPP range (Figure 3A).

NO_2^-

Compared with the control conditions, the amount of NO_2^- released was not changed significantly during the administration of diclofenac or ibuprofen for any CPP value (Figures 3B, 4B).

H_2O_2

Diclofenac induced a significant decrease in H_2O_2 release (Figure 3C) at CPP = 120 cmH_2O, whereas ibuprofen did not significantly affect H_2O_2 release (Figure 4C).

O_2^-

There were no statistically significant changes in O_2^- release during the application of diclofenac or ibuprofen over the entire CPP range (Figures 3D, 4D).

DISCUSSION

The present study aimed to examine the effects of the acute administration of the COX inhibitors diclofenac and ibuprofen on cardiodynamic parameters, coronary autoregulation and oxidative stress biomarkers in isolated rat hearts. This work relates to earlier experiments in our laboratory (23, 12).

In the first part of our study, we focused our attention on the effects of diclofenac and ibuprofen on cardiodynamic parameters as an indicator of myocardial function. Cardiac contractility was estimated by the maximum and minimum rates of LV pressure development (dp/dt max and dp/dt min). Ibuprofen did not cause any changes in myocardial contractility, but diclofenac induced a significant decrease in contractility at higher values of CPP (Figures 1A, 1B, 2A and 2B). Our results related to ibuprofen agreere consistent with the findings of Herbretson et al (24). They showed that ibuprofen does not affect left ventricular contractility (24) in the model of porcine endotoxemia. Moreover, Beamer et al explored the possible effect of ibuprofen on hemodynamic parameters during hypovolemic shock in a canine experimental model, and they also showed that this drug had no influence on cardiac contractility (25). Our results concerning diclofenac are in agreement with Yarishkin et al (13), who concluded that diclofenac may depress cardiac excitability and contractility simultaneously.

One of the possible mechanisms by which diclofenac exhibits depressive effects on the heart is through the reversible inhibition of the Na^+ currents and irreversible inhibition the L-type Ca^{2+} channel currents in cardiac muscle cells (13, 26). Moreover, ibuprofen also exhibits a similar influence (17) on ion channels, but in our results, there was no effect on cardiac contractility.

Both drugs induced decreases in HR and CF at higher CPP values (Figures 1F, 1G, 2F, 2G). Yang et al showed that ibuprofen causes a decrease in HR (17) in the guinea pig isolated heart model. A possible mechanism for this action is decreasing the spontaneous depolarisation rate, thereby slowing the heart rate (17). Diclofenac may also affect the duration of the action potential and heart rate (26). Kristof and co-authors concluded that chronic administration of diclofenac at therapeutic concentrations does not increase the risk of arrhythmia in intact hearts (26). In our experiments, we also used intact hearts, but considering our experimental protocol and acute administration of diclofenac, this mechanism may play a role in decreasing the heart rate.

Although both diclofenac and ibuprofen are classified as nonselective NSAIDs, there are some differences in their COX selectivity; these differences could be a partial cause of their different effects. Namely, ibuprofen is a more potent inhibitor of COX-1 than diclofenac, which is a more selective COX-2 inhibitor (27) (Figure 3). In their meta-analysis, Varas-Lorenzo and co-workers found that diclofenac causes a more increased risk of vascular events compared with ibuprofen (28).

On the other hand, data regarding the influence of these drugs on ROS generation are inconsistent. Li and co-workers demonstrated that diclofenac causes decreased nitrite plasma levels (oxidation product of NO), indicating a reduction in NO bioavailability (11). In the present study, we also found decreased nitrites (Figure 3B). This may lead to vasoconstriction and consequently to a decrease in CF (Figure 1G).

Regarding these findings, we wanted to explore the possible role of oxidative stress in the appearance of side effects with these drugs. However, most of the oxidative

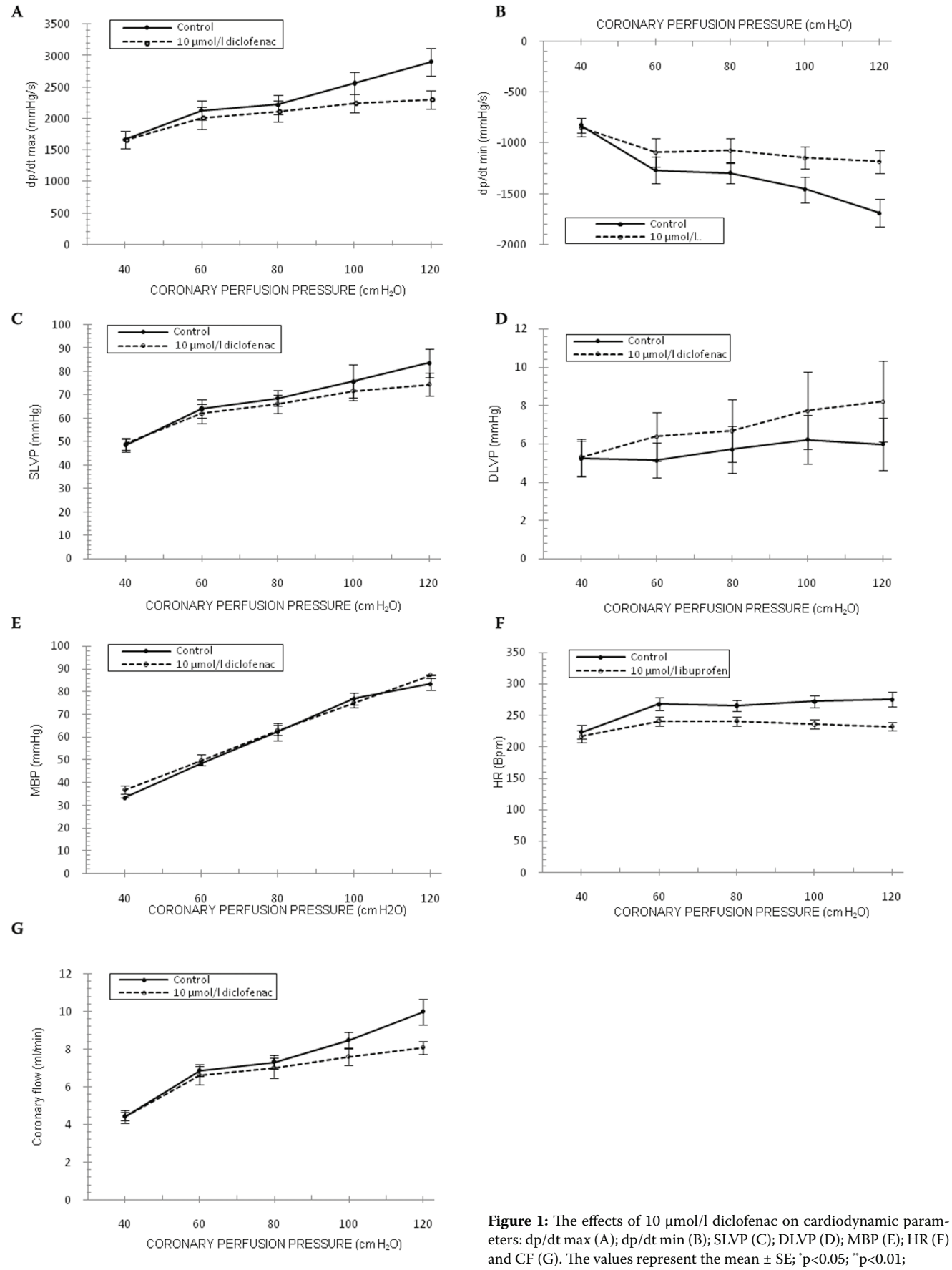

Figure 1: The effects of 10 µmol/l diclofenac on cardiodynamic parameters: dp/dt max (A); dp/dt min (B); SLVP (C); DLVP (D); MBP (E); HR (F) and CF (G). The values represent the mean ± SE; $^{*}p<0.05$; $^{**}p<0.01$;

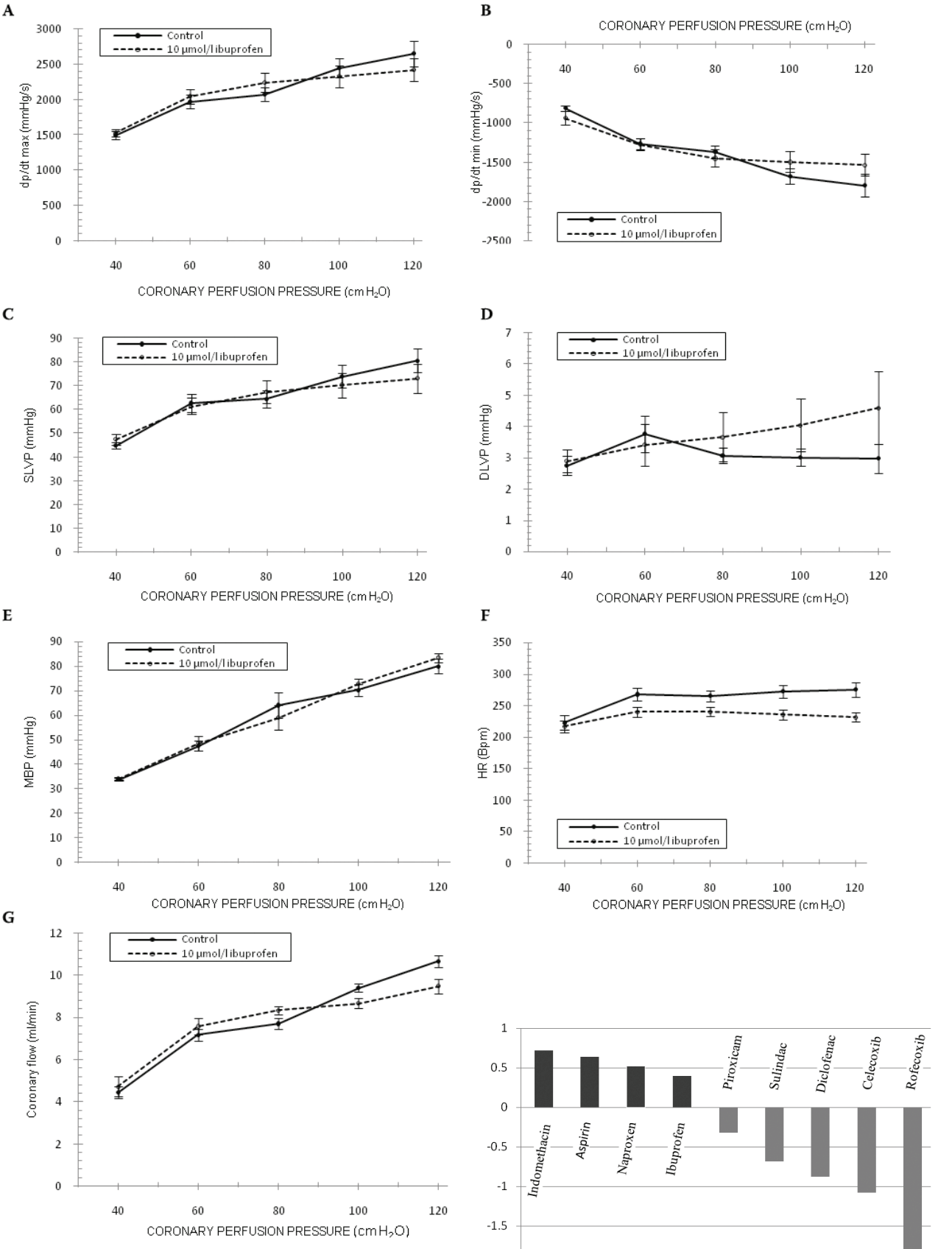

Figure 2: The effects of 10 µmol/l ibuprofen on cardiodynamic parameters: dp/dt max (A); dp/dt min (B); SLVP (C); DLVP (D); MBP (E); HR (F) and CF (G). The values represent the mean ± SE; * p<0.05; ** p<0.01;

Figure 3: COX selectivity of different NSAIDs.

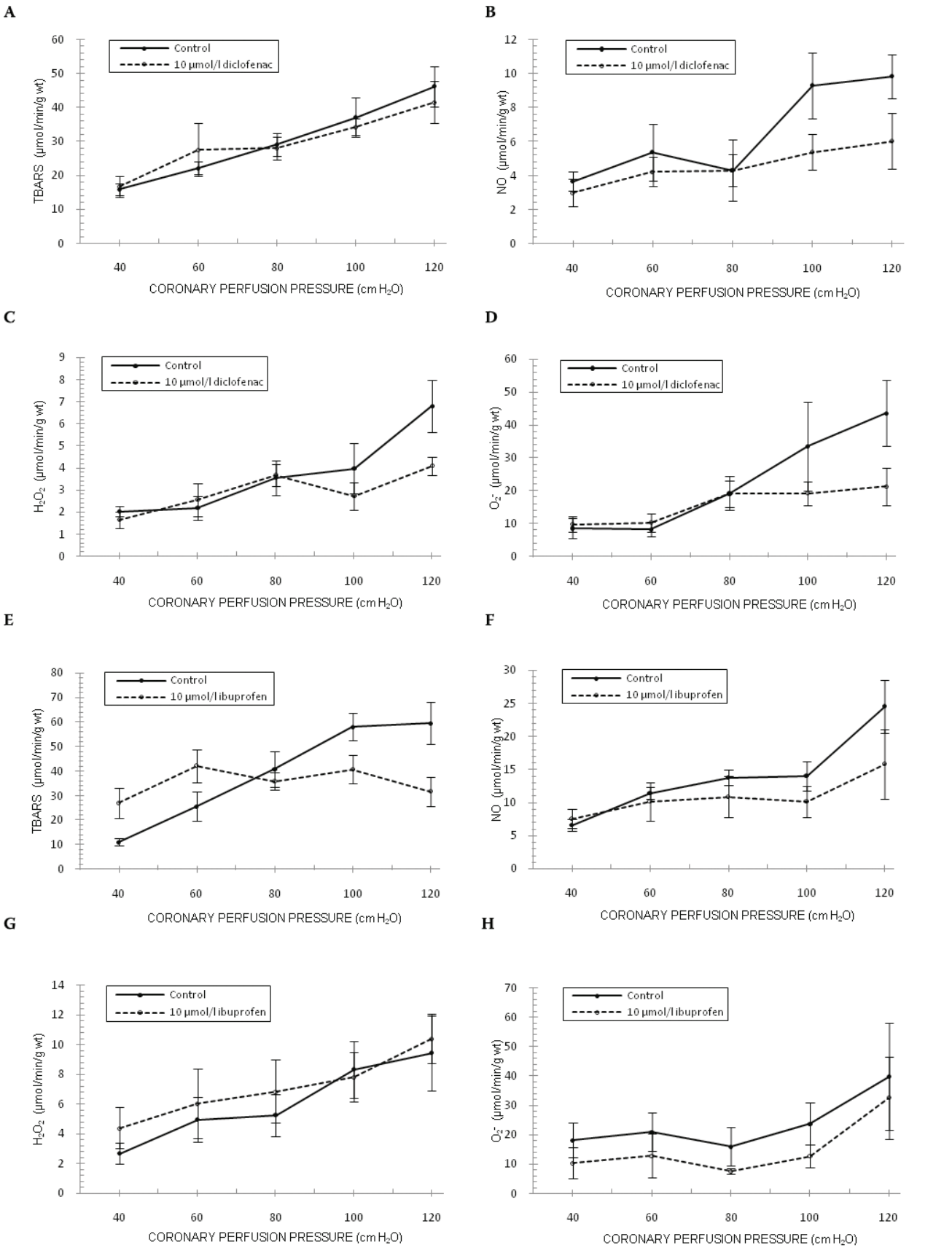

Figure 4: The effects of 10 µmol/l diclofenac and 10 µmol/l ibuprofen on oxidative stress parameters: TBARS diclofenac (A); NO diclofenac (B); H_2O_2 diclofenac (C); O_2^- diclofenac (D); TBARS ibuprofen (E); NO ibuprofen (F); H_2O_2 ibuprofen (G) and O_2^- ibuprofen (H);. The values represent the mean ± SE; $^*p<0.05$; $^{**}p<0.01$;

stress parameters did not change significantly (Figure 3). Diclofenac caused a decrease in H_2O_2 levels at CPP 40 cm H_2O (Figure 3C), and ibuprofen first induced first an increase in TBARS levels at CPP 40 and then a decrease at CPP 100 and 120 (Figure 3E). This result is in contrast with our hypothesis that oxidative stress may be one of the mechanisms by which NSAIDs exhibit their side effects. There are some results suggesting that diclofenac induces a marked increase in vascular ROS content (11). Li et al used spontaneously hypertensive rats and chronic application of NSAIDs (and among them, diclofenac). Differences between the results of our study and theirs may occur because of the different experimental models.

On the other hand, diclofenac may possess some antioxidant potential, behaving as an ROS scavenger (29). Hermann et al found that diclofenac does not improve endothelial dysfunction or affect oxidative stress parameters (30). Wilkinson and his group concluded that ibuprofen attenuates oxidative damage in the brain and has beneficial effects in Alzheimer's disease (31). The findings of these authors suggest that ibuprofen acts independently of COX inhibition to disrupt signalling cascades leading to microglial Nox2 activation, thus preventing oxidative damage. Moreover, the findings of Zhao et al suggest that ROS specifically derived from Nox2 NADPH oxidase make a substantial contribution to several key processes underlying the development of cardiac contractile dysfunction and remodelling (32). In this study, the authors examined the role of Nox2 NADPH oxidase in the development of doxorubicin-induced cardiac injury and remodelling. Considering this, we can suggest that the unchanged levels of oxidative stress biomarkers in our study could be due to the inhibition of Nox2 NADPH oxidase by ibuprofen.

Based on the present data, we can conclude that diclofenac affects cardiodynamic parameters more significantly than does ibuprofen. Furthermore, our results indicate that both estimated COX inhibitors do not promote the production of ROS; therefore, their cardiac effects do not appear to be mediated via oxidative stress, which could be important in elucidating their potential deleterious influence on cardiac muscle and coronary endothelium.

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