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RESEARCH PAPER

The hydrogen sulphide-releasing derivative of diclofenac protects against ischaemia–reperfusion injury in the isolated rabbit heart

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Background and purpose: Hydrogen sulphide (H_2S) is an endogenous gaseous mediator active in the multilevel regulation of pathophysiological functions in mammalian cardiovascular tissues.

Experimental approach: This study investigated the pharmacological activity of a new H_2S-releasing derivative of diclofenac, S-diclofenac (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester) in the isolated rabbit heart submitted to low-flow ischaemia-reperfusion damage.

Key results: S-diclofenac (3, 10 and 30 μM), despite inhibiting prostacyclin generation by cardiac tissues, achieved dose-dependent normalization of coronary perfusion pressure, reducing left ventricular contracture during ischaemia and improving left ventricular developed pressure and $\pm dP/dt_{max}$ at reperfusion. Creatine kinase and lactate dehydrogenase activities in heart perfusates were significantly reduced during reperfusion. These effects were accompanied by substantial release of reduced glutathione (GSH), indicating that the H_2S moiety may have up-regulated cysteine transport. The anti-ischaemic activities of S-diclofenac and the H_2S-donor sodium hydro sulphide (NaHS) were partially prevented by the K_{ATP} channel antagonist glibenclamide, suggesting a mechanism similar to H_2S-induced cardioprotection in metabolic ischaemic preconditioning. Perfusion with the nitric oxide (NO) synthase inhibitor N^G-monomethyl-L-arginine worsened the myocardial ischaemia-reperfusion damage, but this was dose-dependently prevented by S-diclofenac and NaHS, suggesting that the released H_2S may have overcome NO deficiency.

Conclusion and implications: These data show that S-diclofenac had marked anti-ischaemic activity in ischaemic-reperfused rabbit hearts despite inhibition of prostaglandin generation. Increased GSH formation leading to activation of K_{ATP} channels may have contributed to this beneficial effect. The pharmacological profile of S-diclofenac and its anti-inflammatory activity, with diminished gastrointestinal side effects, offer therapeutic applications in cardiovascular disease.

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Abbreviations: 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; AUC, area under the curve; CBS, cystathionine β-synthase; CK, creatine kinase; CPP, coronary perfusion pressure; CSE, cystathionine γ-lyase; $\pm dP/dt_{max}$, maximum rate of rise and fall of left ventricular pressure; DMSO, dimethylsulphoxide; GSH, reduced glutathione; KHS, Krebs Henseleit solution; K_{ATP}, ATP-dependent K^+ channel; LDH, lactate dehydrogenase; L-NMMA, N^G-monomethyl-L-arginine; LVP, left ventricular pressure; LVDevP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; NaHS, sodium hydrosulphide; PGI_2, prostacyclin; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester

Introduction

Hydrogen sulphide (H_2S), like nitric oxide (NO) and carbon monoxide, is an endogenous gaseous mediator active in the multilevel regulation of physiological and pathological functions in mammalian tissues (Li *et al*., 2006; O'Sullivan, 2006). Synthesis of H_2S from cysteine occurs naturally through the activity of two pyridoxal-5′-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), although other sources cannot be ruled out (Boehning and Snyder, 2003; Moore *et al*., 2003; Stipanuk, 2004). The localization of the H_2S synthesizing enzymes and

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their endogenous levels in tissues imply that the cardiovascular system is a source of endogenous H_2S. CSE appears to be the main source of H_2S in the vasculature and heart (Wang, 2002), but CBS predominates in control of the nervous system (Boehning and Snyder, 2003). mRNA for the H_2S producing enzyme CSE is expressed in myocardial tissues and H_2S might also be produced in these tissues where it acts as a physiological regulator of cardiac function, mediated by the ATP-dependent K^+ channel (K_{ATP}) pathway (Geng *et al*., 2004). Johansen *et al*. (2006) have provided the first evidence that exogenous H_2S protects against irreversible ischaemia–reperfusion injury in the rat myocardium, supporting the likelihood of K_{ATP} opening in the mechanism of action. Elrod *et al*. (2007), working with an *in vivo* murine model of myocardial ischaemia–reperfusion injury, reported that the H_2S-donor sodium hydrosulphide (NaHS) achieved significant cardioprotection through reversible inhibition of mitochondrial respiration at the time of reperfusion, thereby reducing oxidant generation and cardiac apoptosis.

On the grounds that H_2S may play a beneficial role in inflammation (Zanardo *et al*., 2006) and that the inhibition of the generation of this gaseous transmitter contributes to gastric injury caused by non-steroidal anti-inflammatory drugs (Fiorucci *et al*., 2005), a novel H_2S-releasing derivative of diclofenac (*S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]-benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester), has been recently synthesized (Bhatia *et al*., 2005; Li *et al*., 2007). *S*-diclofenac shows potent anti-inflammatory activity with less gastric intolerance that the parent compound, with a significant release of H_2S (Li *et al*., 2007).

In the light of this information, and considering that H_2S and NO may react together to form a molecule (possibly a nitrosothiol) that might regulate NO availability in the cardiovascular system (Ali *et al*., 2006), we compared the effects of *S*-diclofenac and diclofenac in the isolated rabbit heart submitted to low-flow ischaemia–reperfusion. To gain more information on the relationship between H_2S and NO, we studied the cardioprotective activity of *S*-diclofenac in the perfused heart submitted to low-flow ischaemia–reperfusion in the presence of the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA) (Rossoni *et al*., 1995). Previous work in rats and rabbits with NO-releasing aspirin (Rossoni *et al*., 2000, 2001) and NO-releasing naproxen (Rossoni *et al*., 2004) indicated significant cardioprotection in ischaemia–reperfusion experiments compared to the parent compounds.

Materials and methods

Animals

All animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male New Zealand White rabbits (Charles River Laboratories, Calco, Lecco, Italy) weighing 1.9–2.1 kg were used. The animals were housed in a conditioned environment (22 ± 1 °C, 55 ± 5% relative humidity, 12-h light/12-h dark cycle), with free access to food and tap water. At least 5 days were allowed for animals to acclimatize before any experimental manipulations.

Rabbit heart perfusion

Rabbit hearts were perfused as described previously (Henry *et al*., 1977; Rossoni *et al*., 2000, 2004). In brief, the rabbits were anaesthetized with thiopentone sodium (Pentothal; Abbott, Campoverde, Latina, Italy, 60 mg per kg) given by i.v. injection. The chest was opened, and the heart was rapidly excised and placed in cold (4 °C) Krebs Henseleit solution (KHS) with the following composition (mM): NaCl 118, KCl 4.8, KH_2PO_4 1.2, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.5. The heart, mounted on the experimental setup, was perfused retrogradely through the aorta at 20 ml min^{-1} (Minipuls-3 peristaltic pump; Gilson, Villiers-Le Bel, France) with KHS, which was held at 37 °C and aerated with 95% O_2 + 5% CO_2 to maintain normal pH, pO_2 and pCO_2 parameters. Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were measured with two HP-1280C pressure transducers (Hewlett-Packard, Waltham, MA, USA) connected to a Hewlett-Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter with a small latex balloon on the tip (Hugo Sachs Elektronik, March-Hugstetten, Germany), inserted in the left ventricular cavity through the mitral valve opening. The volume of the balloon was adjusted to give peak left ventricular systolic pressure (LVSP) 97–100 mm Hg and left ventricular end-diastolic pressure (LVEDP) of 3- to 5-mm Hg. Hearts that could not achieve this contractile performance (7–8% of the hearts) were excluded. Left ventricular developed pressure (LVDevP; peak LVSP minus LVEDP) and the maximum rate of rise and fall of left ventricular pressure ($\pm dP/dt_{max}$) were also calculated.

Myocardial ischaemia–reperfusion

After 15 min equilibration, hearts were paced at 180 beats min^{-1} (Henry *et al*., 1977) with an electrical stimulator (S-88; Grass Instr., Quincy, MA, USA) using two silver electrodes attached to the right atrium and an additional 30 min of perfusion was carried out (pre-ischaemic period). Ischaemia was induced by reducing the flow rate from 20 to 1 ml min^{-1} for 40 min (ischaemic period). Normal flow rate (20 ml min^{-1}) was then restored and the perfusion was continued for another 20 min (reperfusion period). Throughout the experiment, a thermoregulated chamber kept the heart temperature at 37 °C to avoid hypothermia-induced cardioprotection. Each experiment did not last for more than 2 h, during which time the experimental preparation was stable.

Experimental design

After preliminary experiments, different molar concentrations of the compounds under investigation were selected and tested in groups of seven hearts each. In particular, *S*-diclofenac (3, 10 and 30 μM) or diclofenac (3, 10 and 30 μM) were perfused through the hearts for 20 min before coronary flow was reduced. In some experiments ($n = 6$ hearts per group) we compared the effect of *S*-diclofenac (30 μM) with the H_2S-donor NaHS (30 μM), infused through the hearts for 20 min before flow reduction. The K_{ATP} inhibitor glibenclamide (100 μM) was given through the hearts for 30 min

during the pre-ischaemic period; this infusion started 10 min before treatment with *S*-diclofenac or NaHS.

Creatine kinase and lactate dehydrogenase activities in heart perfusates
The perfusates, eluted from the heart during pre-ischaemic and reperfusion periods, were collected in an ice-cooled beaker, as 5 min samples. Each sample was used for the determination of creatine kinase (CK) and lactate dehydrogenase (LDH) activities according to the original method of Bergmeyer *et al*. (1970) and Hohorst (1963), respectively. The total activity of these enzymes was measured spectrophotometrically (Lambda-16; Perkin Elmer Italia, Monza, Milan, Italy) at 37 °C using specific kits, according to the manufacturer's instructions. Data are expressed as mU min^{-1} per g wet tissue (w.t.).

Prostacyclin and reduced glutathione determinations in heart perfusates
Prostacyclin (PGI_2) and reduced glutathione (GSH) were measured directly in the coronary effluent collected in an ice-cooled beaker for 2 min immediately before ischaemia and during the first 10 min of reperfusion. PGI_2 was determined as its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) with an ELISA kit (detection limit, 3 pg ml^{-1}) as described by Pradelles *et al*. (1985). GSH formation was analysed using a commercial GSH enzymatic assay kit (Anderson, 1985). PGI_2 and GSH were assayed in duplicate and the results were expressed as ng min^{-1} per g w.t. and nnol min^{-1} per g w.t., respectively.

Myocardial ischaemia–reperfusion experiments with NOS inhibition: effects of S-diclofenac, diclofenac and NaHS
In this set of experiments ($n = 5$ hearts per group) hearts were perfused for 10 min immediately before ischaemia with the NOS inhibitor, L-NMMA (10 μM). Previous papers (Rossoni *et al*., 1995, 2000, 2004) reported that at this regimen L-NMMA increases CPP, aggravating post-ischaemic ventricular dysfunction. To evaluate cardioprotective activity, *S*-diclofenac, diclofenac and NaHS were perfused through the hearts at the concentration of 30 μM for 20 min (10 min before L-NMMA plus 10 min during L-NMMA).

Statistical analysis
Each value is the mean ± s.e.mean. Statistical significance was established by ANOVA followed by Bonferroni's multiple comparisons. Differences with a probability of 5% or less were considered significant. The area under the curve (AUC) was estimated according to the trapezoid method (Purves, 1992) using the Microcal Origin 3.5 computer program (Microcal Software Inc., Northampton, MA, USA).

Drugs
The following drugs were used: *S*-diclofenac (CTG Pharma, Milan, Italy), diclofenac sodium salt, glibenclamide, NaHS and L-NMMA (Sigma-Aldrich, St Louis, MO, USA), assay kits for CK and LDH (Sentinel Diagnostic, Milan, Italy) and for 6-keto-$PGF_{1\alpha}$ and GSH determinations (Cayman Chemical Co., Ann Arbor, MI, USA). NaHS (containing 28–32% H_2O) was dissolved freshly in ultrapure water to provide a stock solution of 1 mM NaHS. *S*-diclofenac, diclofenac and glibenclamide, dissolved in dimethylsulphoxide (DMSO) at 0.5 M stock concentration and further diluted in KHS, were prepared daily. The final DMSO concentration (<0.1%) (vehicle) *per se* had no effects on the parameters tested. All other chemicals were of analytical or ultrapure grade.

Results

Ischaemia–reperfusion in isolated rabbit heart
At baseline, the cardiac parameters were similar and not significantly different ($P > 0.05$) in the seven experimental groups (Table 1). When the perfusion of electrically paced isovolumic left rabbit heart preparations was reduced from 20 to 1 ml min^{-1} for 40 min, LVEDP progressively rose indicating that, after the standstill, an ischaemic process was occurring. During reperfusion left ventricular function was impaired, LVDevP and $+dP/dt_{max}$ being significantly reduced, and CPP considerably increased from baseline (Figures 1–4). Perfusion of the hearts with *S*-diclofenac (3, 10 and 30 μM) for 20 min before ischaemia gave dose-dependent myocardial protection against mechanical changes due to the ischaemia–reperfusion. The characteristic ventricular contracture observed during the 40 min of ischaemia was reduced, favouring a better recovery of

Table 1 Cardiac parameters determined in isolated perfused rabbit hearts at baseline

Treatment	CPP (mm Hg)	LVEDP (mm Hg)	LVDevP (mm Hg)	$+dP/dt_{max}$ (mm Hg s^{-1})	$-dP/dt_{max}$ (mm Hg s^{-1})
Vehicle	58.2 ± 6.3	3.6 ± 0.5	93.4 ± 5.7	2179 ± 185	1672 ± 215
S-diclofenac (3 μM)	56.7 ± 6.1	3.3 ± 0.4	95.2 ± 4.8	2238 ± 201	1698 ± 185
S-diclofenac (10 μM)	61.8 ± 4.9	3.7 ± 0.3	88.7 ± 7.0	1987 ± 164	1592 ± 149
S-diclofenac (30 μM)	59.1 ± 5.5	3.3 ± 0.3	91.0 ± 5.1	2117 ± 173	1613 ± 201
Diclofenac (3 μM)	56.4 ± 3.7	4.1 ± 0.6	92.5 ± 6.3	2091 ± 204	1644 ± 197
Diclofenac (10 μM)	62.0 ± 4.3	3.7 ± 0.3	89.1 ± 7.1	2275 ± 192	1780 ± 180
Diclofenac (30 μM)	60.8 ± 5.0	4.3 ± 0.5	97.6 ± 6.0	2256 ± 217	1742 ± 238

Abbreviations: CPP, coronary perfusion pressure; LVDevP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; $+dP/dt_{max}$ and $-dP/dt_{max}$, maximum rate of rise and fall of left ventricular pressure; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester; w.t., wet tissue.
Data are mean ± s.e.mean of seven heart preparations per group. Cardiac parameters were calculated immediately before ischaemia.

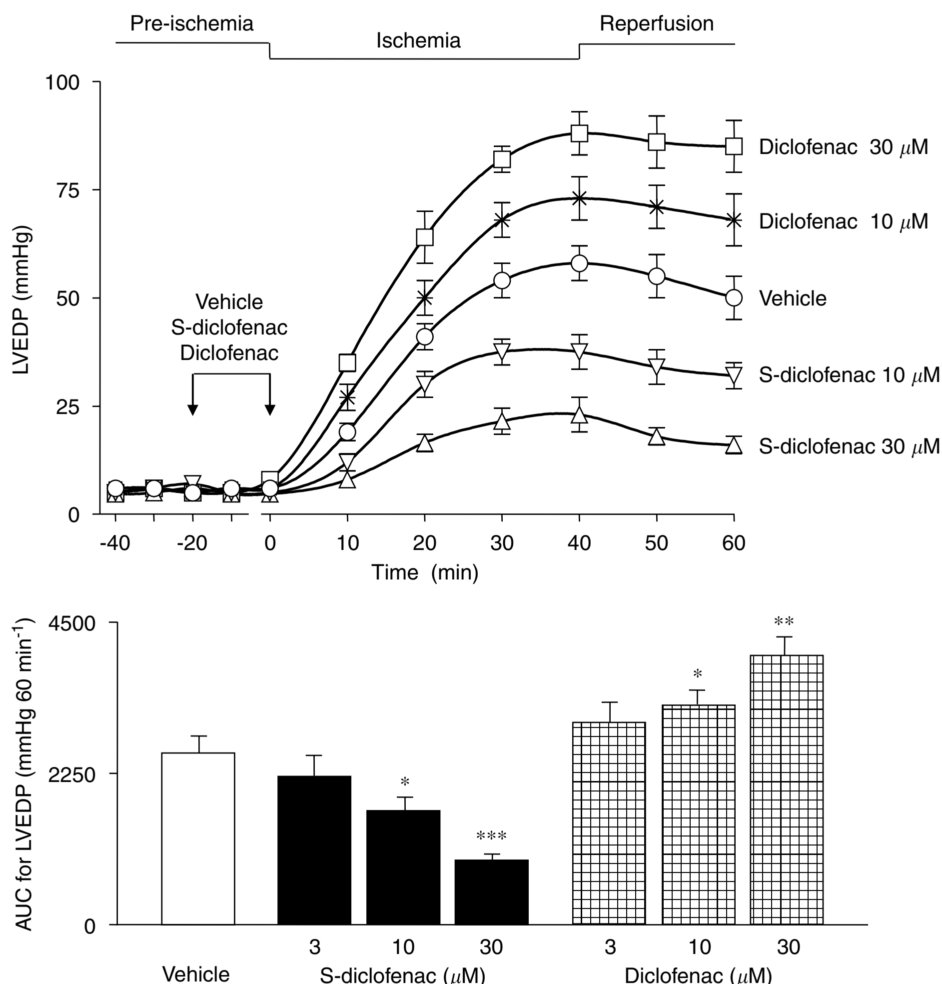

Figure 1 Effects of *S*-diclofenac and diclofenac on LVEDP in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia–reperfusion. The upper panel show the time-course of activity for two drug concentrations, and the bar graph (lower panel) shows the AUC for all concentrations of each drug. Each point/bar represents the mean ± s.e.mean of seven experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle group. AUC, area under the curve; LVEDP, left ventricular end-diastolic pressure; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]-benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

LVDevP and $+dP/dt_{max}$ at reperfusion (Figures 1–3). At the same time, CPP fell in proportion to the *S*-diclofenac dose (Figure 4). In clear contrast with this picture, perfusion with diclofenac (3, 10 and 30 μM) severely worsened the myocardial ischaemic damage. At reperfusion, the LVEDP was significantly higher than in vehicle-treated preparations, and this was associated with a marked depression of LVDevP and $+dP/dt_{max}$, and an increase in CPP (Figures 1–4). *S*-diclofenac and diclofenac had no effects on LVEDP, LVDevP, $+dP/dt_{max}$ and CPP values during the pre-ischaemic period (Table 1).

CK and LDH activities in heart perfusates

CK and LDH, as indicators of myocardial damage, were determined in the coronary effluent collected from each heart during the pre-ischaemic and reperfusion periods. There were no differences in the pre-ischaemic period (data not shown). During reperfusion, CK and LDH activities in the vehicle-treated group were 5.2 times ($P<0.001$) and 9.3 times ($P<0.001$) those in the pre-ischaemic period (CK, 0.92 ± 0.16 U per g w.t. per 20 min; LDH, 1.75 ± 0.33 U per g w.t. per 20 min) (data not shown). *S*-diclofenac (3, 10 and 30 μM), perfused for 20 min before ischaemia, reduced the release of CK and LDH in a concentration-dependent manner at reperfusion, compared to vehicle-treated hearts (Figure 5). Unlike the effects of *S*-diclofenac, the increased severity of post-ischaemic ventricular dysfunction caused by diclofenac was associated with a marked increase of both CK and LDH activities in heart effluents (Figure 5).

PGI_2 release in heart perfusates

PGI_2 is the major eicosanoid produced by jeopardized myocardium (Van Bilsen *et al.*, 1989) and its rate of formation increases particularly during the first 5–10 min of reperfusion, declining rapidly thereafter (Berti *et al.*, 1988; Engels *et al.*, 1990). In the present study, in vehicle-treated hearts the generation of 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2) during reperfusion was enhanced 4.2 times

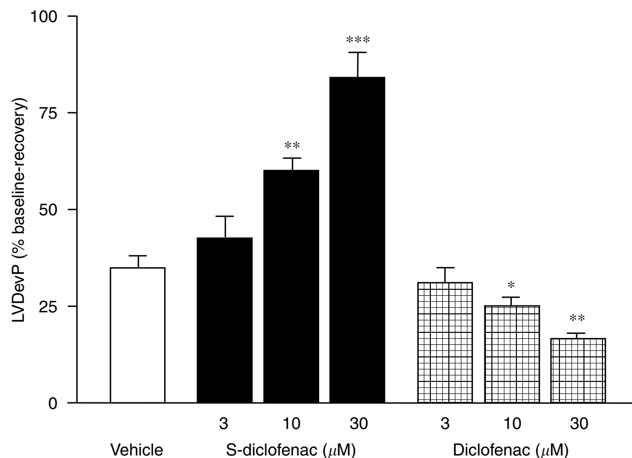

Figure 2 Effects of *S*-diclofenac and diclofenac on LVDevP in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia and reperfusion. LVDevP was calculated at the end of the 20-min reperfusion and expressed as percentage of the pre-ischaemic values (see Table 1). Each bar represents the mean ± s.e.mean of seven experiments.*$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle group. LVDevP, left ventricular developed pressure; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

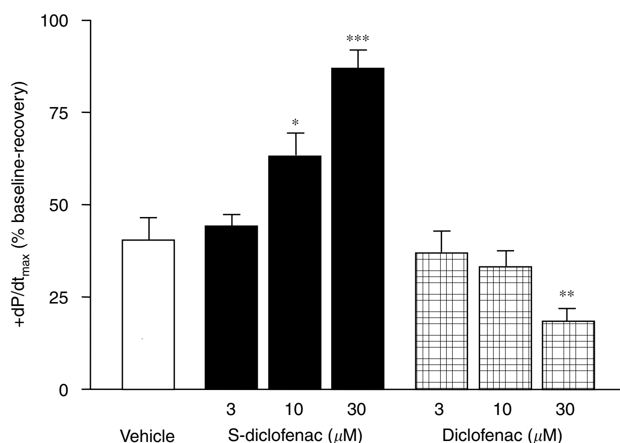

Figure 3 Effects of *S*-diclofenac and diclofenac on $+dP/dt_{max}$ in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia and reperfusion. $+dP/dt_{max}$ was calculated at the end of the 20-min reperfusion, and expressed as percentage of the pre-ischaemic values (see Table 1). Each bar represents the mean ± s.e.mean of seven experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle group. $+dP/dt_{max}$, maximum rate of rise of left ventricular pressure; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

($P<0.001$) compared to the pre-ischaemic period ($3.15 \pm 0.53\,ng\,min^{-1}$ per g w.t.) (Table 2). When the hearts were perfused with *S*-diclofenac or diclofenac, 6-keto-$PGF_{1\alpha}$ release was inhibited in a concentration-dependent manner in both the pre-ischaemic and reperfusion periods. Diclofenac inhibited 6-keto-$PGF_{1\alpha}$ release at a concentration approximately three times lower than that required to obtain a similar inhibition with *S*-diclofenac (Table 2).

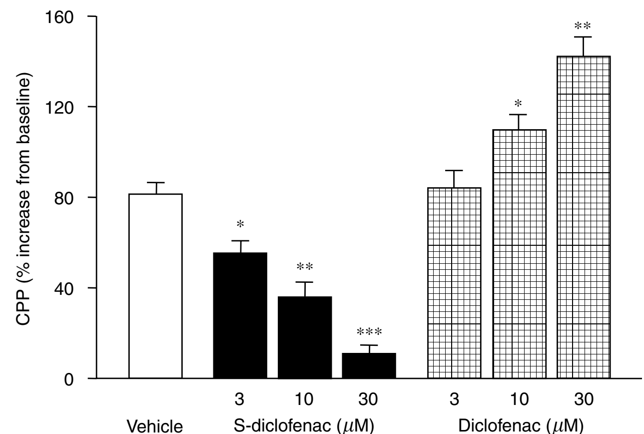

Figure 4 Effects of *S*-diclofenac and diclofenac on CPP in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia–reperfusion. CPP was calculated at the end of the 20-min reperfusion, and expressed as percentage of the pre-ischaemic values (see Table 1). Each bar represents the mean ± s.e.mean of seven experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle group. CPP, coronary perfusion pressure; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

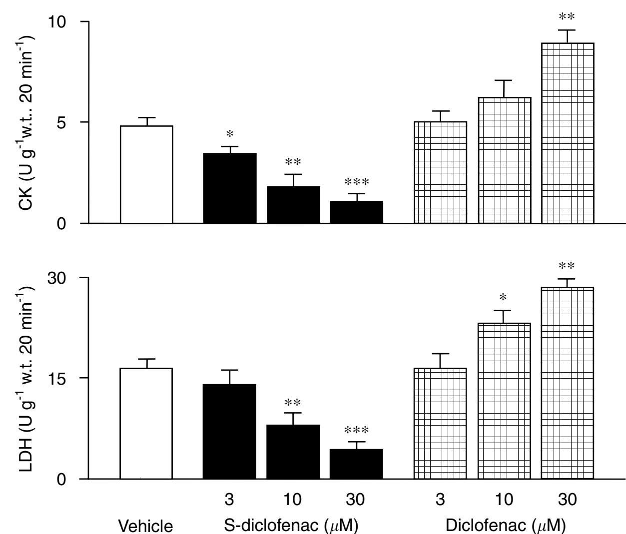

Figure 5 Effects of *S*-diclofenac and diclofenac on CK and LDH activities in paced isovolumic left rabbit heart preparations submitted to low-flow ischaemia–reperfusion. CK and LDH were calculated during the 20-min reperfusion and expressed as the increase from pre-ischaemic values (CK, 0.92 ± 0.16 U per g w.t. per 20 min; LDH, 1.75 ± 0.33 U per g w.t. per 20 min). Each bar represents the mean ± s.e.mean of seven experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle group. CK, creatine kinase; LDH, lactate dehydrogenase; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

GSH release in heart perfusates

The results for GSH in heart perfusates are shown in Table 3. In vehicle-treated hearts, the GSH was 9.8 times the pre-ischaemic level at reperfusion. Perfusion with *S*-diclofenac caused a concentration-dependent increase of GSH in the

Table 2 Effects of *S*-diclofenac and diclofenac on 6-keto-PGF$_{1\alpha}$ formation in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia and reperfusion

Treatment		*6-keto-PGF$_{1\alpha}$ (ng min^{-1} per g w.t.)*	
		Pre-ischaemia	*Reperfusion*[a]
Vehicle		3.15 ± 0.53	13.22 ± 1.42
S-diclofenac	3 μM	2.38 ± 0.37 (24)	10.81 ± 1.05 (18)
S-diclofenac	10 μM	1.48 ± 0.29 ** (53)	7.13 ± 0.94 * (46)
S-diclofenac	30 μM	0.51 ± 0.12 *** (84)	3.21 ± 0.51 *** (76)
Diclofenac	3 μM	1.61 ± 0.32 ** (49)	8.33 ± 1.04 * (37)
Diclofenac	10 μM	0.57 ± 0.10 *** (82)	3.59 ± 0.18 *** (73)
Diclofenac	30 μM	n.d.	0.96 ± 0.23 *** (93)

Abbreviations: 6-keto-PGF$_{1\alpha}$, 6-keto-prostaglandin F$_{1\alpha}$; n.d., not detectable (detection limit 3 pg ml^{-1}); *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester; w.t., wet tissue.
Data are mean ± s.e.mean of seven heart preparations per group. Values within parentheses show, percentage inhibition compared to vehicle-treated hearts. Drugs were infused for 20 min before flow rate reduction.
[a]Data refer to the first 10 min of reperfusion.
*$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle-treated hearts.

Table 3 Effects of *S*-diclofenac and diclofenac on reduced GSH formation in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia and reperfusion

Treatment		*GSH (nmol min^{-1} per g w.t.)*	
		Pre-ischaemia	*Reperfusion*[a]
Vehicle		0.53 ± 0.06	5.18 ± 0.67
S-diclofenac	3 μM	0.49 ± 0.06 (−8)	5.53 ± 0.44 (+7)
S-diclofenac	10 μM	0.66 ± 0.11 (+24)	7.15 ± 0.62* (+38)
S-diclofenac	30 μM	0.81 ± 0.09* (+52)	8.29 ± 0.49** (+60)
Diclofenac	3 μM	0.57 ± 0.10 (+7)	5.07 ± 0.30 (−2)
Diclofenac	10 μM	0.52 ± 0.06 (−2)	4.61 ± 0.56 (−11)
Diclofenac	30 μM	0.46 ± 0.05 (−13)	3.98 ± 0.47 (−23)

Abbreviations: GSH, glutathione; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino] benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester; w.t., wet tissue.
Data are mean ± s.e.mean of seven heart preparations per group. Values in the parentheses show, percentage from vehicle-treated hearts. Drugs were infused for 20 min before flow rate reduction.
[a]Data refer to the first 10 min of reperfusion.
*$P<0.05$ and **$P<0.01$ versus vehicle-treated hearts.

perfusate. This was particularly marked at 30 μM, when *S*-diclofenac significantly raised the rate of formation of GSH in both the pre-ischaemic and reperfusion periods. In contrast, diclofenac did not affect GSH release at any concentration (Table 3).

S-diclofenac and NaHS activity in myocardial ischaemia–reperfusion experiments: effect of glibenclamide
In these experiments, perfusion of the hearts with *S*-diclofenac (30 μM) and NaHS (30 μM) for 20 min before ischaemia clearly protected the myocardium, and the AUC for LVEDP were respectively 66% ($P<0.001$) and 52% ($P<0.001$) lower for *S*-diclofenac and NaHS than for vehicle-treated preparations (AUC = 2265 ± 196 mm Hg 60 min^{-1}) (Figure 6). Pretreatment with 100 μM glibenclamide, which *per se* did not affect LVEDP, significantly antagonized the cardioprotecting activity of *S*-diclofenac and NaHS. The AUC for LVEDP in preparations treated with *S*-diclofenac and NaHS plus glibenclamide were only 23% ($P<0.05$) and 14% lower than the vehicle-treated preparations (Figure 6).

Effects of S-diclofenac, diclofenac and NaHS in myocardial ischaemia–reperfusion experiments with NOS inhibition
Perfusion of the hearts with L-NMMA (10 μM) for 10 min before flow reduction exacerbated ventricular dysfunction compared with vehicle-treated preparations. At the end of the ischaemic period, LVEDP rose to nearly twice the values in vehicle-treated hearts; $P<0.001$). At the end of reperfusion these values were still high (Figure 7) and cardiac contractility was severely depressed with a significant rise of CPP (data not shown). In these preparations, where the myocardial ischaemic damage was aggravated by L-NMMA, pretreatment with *S*-diclofenac (30 μM) or NaHS (30 μM) significantly prevented the worsening effects of NOS inhibition (Figure 7). *S*-diclofenac and NaHS reduced the AUC for the LVEDP increase by respectively 73% ($P<0.01$) and 67% ($P<0.01$) compared to L-NMMA-alone preparations (Figure 6). Diclofenac (30 μM) did not affect the increased myocardial–ischaemia injury due to L-NMMA (Figure 7).

Discussion

The main findings of this study indicate that the H_2S-releasing derivative of diclofenac, *S*-diclofenac, provided marked cardioprotection in a well-characterized experimental model of myocardial ischaemia–reperfusion injury in the rabbit (Henry *et al*., 1977; Rossoni *et al*., 2000, 2004). In this model, the major determinants of myocardial performance are under control, and changes in $+dP/dt_{max}$ and LVEDP are used as indexes of cardiac contractility and diastolic elastic stiffness ('compliance'), respectively (Henry *et al*., 1977).

Unlike diclofenac, *S*-diclofenac reduced LVEDP, consequently improving LVDevP and $+dP/dt_{max}$, and lowering CPP at reperfusion. In spite of dose-related impairment of PGI_2 formation by cardiac endothelial cells, the beneficial effects of *S*-diclofenac were accompanied by dose-dependent reduction of both CK and LDH activities in the cardiac perfusates, suggesting there was less loss of functional integrity of the sarcolemma. This may imply that H_2S released by *S*-diclofenac is responsible for the drug's cardioprotective activity in the ischaemic–reperfused rabbit heart. However, we did not directly determine H_2S released by *S*-diclofenac in cardiac tissues or in heart perfusates and this is now under investigation. It has been reported that *in vitro* incubation of 100 μM *S*-diclofenac with rat liver homogenate resulted in H_2S release into the medium which peaked at 15 min (2.6 ± 0.1% of substrate added) and remained elevated for a further 75 min (Li *et al*., 2007). Indirect evidence for H_2S release from *S*-diclofenac appears to be the enhanced formation of GSH, particularly marked when *S*-diclofenac was perfused through the hearts at the maximal cardioprotective dose of 30 μM. It is worth noting that, in a well-studied model of oxidative stress caused by

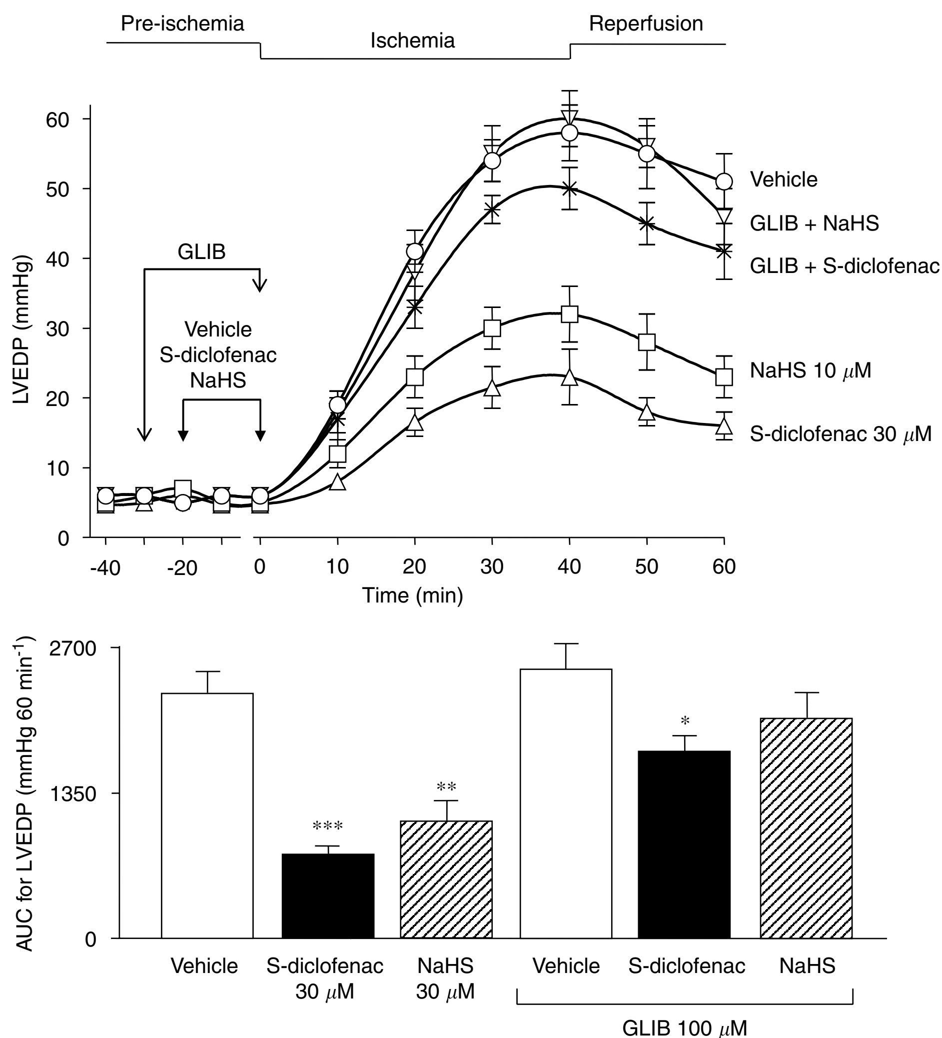

Figure 6 Glibenclamide (GLIB) antagonized the cardioprotective effects of both *S*-diclofenac and NaHS on LVEDP in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia–reperfusion. The upper panel shows the time-course of activity of the drugs, and the bar graph (lower panel) the AUC. Each point/bar represents the mean ± s.e.mean of six experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus the corresponding vehicle group. AUC, area under the curve; LVEDP, left ventricular end-diastolic pressure; NaHS, sodium hydrosulphide; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

glutamate, H_2S protected primary cultures of neurons from death by raising glutathione levels through upregulation of cystine transport, cystine being the rate-limiting substrate of glutathione synthesis (Kimura and Kimura, 2004).

In the present experiments, the increased formation of GSH may have contributed to *S*-diclofenac's beneficial effect against ischaemia–reperfusion damage. GSH, with its important antioxidant properties, plays a pivotal role in myocardial protection against ischaemia–reperfusion (Pan *et al*., 2006). Under conditions of oxidative stress, GSH reacts either as an electron donor to neutralize hydrogen peroxides and lipoperoxides or as a direct free radical scavenger (Leichtweis and Ji, 2001). Interestingly, in vehicle-treated preparations GSH generation was several times higher than the basal values during reperfusion, suggesting a protective mechanism against free radical production generated by cardiac tissues. In these conditions, diclofenac did not change the basal rate of GSH formation, and the worsening of myocardial ischaemia–reperfusion injury caused by this anti-inflammatory drug appears to be related to the inhibition of COX activity, with impaired formation of PGI_2.

Prostaglandin formation, namely PGI_2, is involved in a critical cytoprotective mechanism against myocardial damage caused by ischaemia (Ogletree *et al*., 1979) and the rate of PGI_2 formation in the ischaemic–reperfused rabbit heart increases with the severity of the ischaemic process (Berti *et al*., 1988). Stabilization of cardiac lysosomes by normal PGI_2 generation is of paramount importance in the ischaemic myocardium, because leakage of lysosomal enzymes (proteases and phospholipases) may contribute to the generation of irreversible damage in cardiomyocytes (Wildenthal *et al*., 1978).

The non-selective K_{ATP} blocker glibenclamide partially antagonized the beneficial effects of both *S*-diclofenac and NaHS in myocardial ischaemia–reperfusion damage. This seems to indicate that H_2S, released in sufficient amounts by

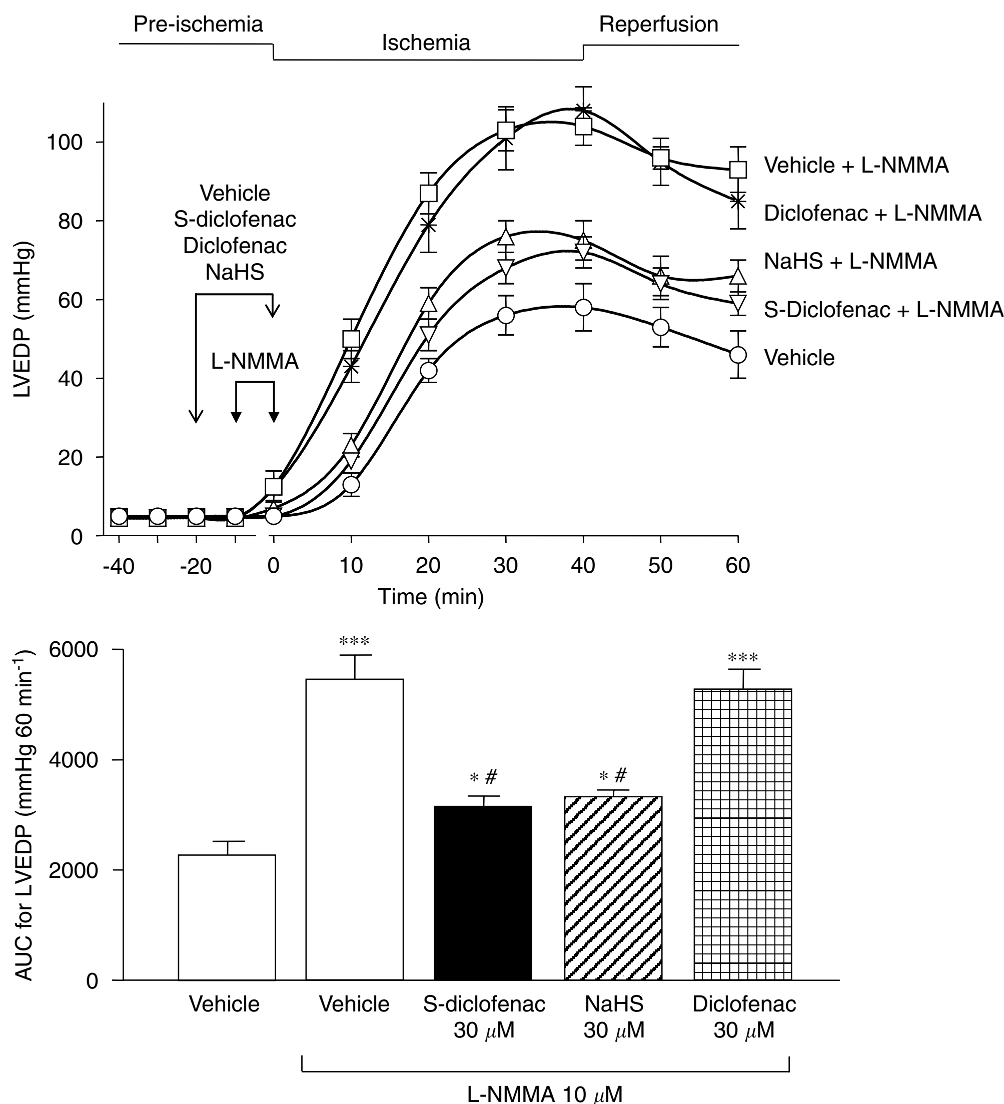

Figure 7 *S*-diclofenac and NaHS counteract the worsening induced by L-NMMA on LVEDP in paced isovolumic left rabbit heart preparations subjected to low-flow ischaemia–reperfusion. The upper panel shows the time-course of activity of the drugs, and the bar graph (lower panel) the AUC. Each point/bar represents the mean ± s.e.m. of five experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus vehicle alone group. $^{\#}P<0.01$ versus L-NMMA alone group. AUC, area under the curve; L-NMMA, N^G-monomethyl-L-arginine; LVEDP, left ventricular end-diastolic pressure; NaHS, sodium hydrosulphide; *S*-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester.

both *S*-diclofenac and NaHS, not only may have overcome the reduced production of H_2S in ischaemic cardiomyocytes (Lapenna *et al*., 1996), but may have also triggered a signalling mechanism similar to that described for metabolic ischaemic preconditioning, where activation of sarcolemmal (and not mitochondrial) K_{ATP} play an important role (Gross and Peart, 2003; Bian *et al*., 2006; Johansen *et al*., 2006). However, additional mechanism/s involved in the cardioprotection provided by *S*-diclofenac may not be ruled out.

Recent findings that NO may regulate H_2S production (Zhao *et al*., 2003; Zhong *et al*., 2003) strongly suggest an interaction between NO and the H_2S system in metabolic preconditioning ischaemia. The results from the present study clearly support the concept that both H_2S and NO are also involved in post-ischaemic ventricular dysfunction. The aggravation of ischaemia–reperfusion damage in rabbit heart preparations, with impaired NO generation caused by L-NMMA, was prevented by both *S*-diclofenac and NaHS. Reports that H_2S prevents the development of hypertension induced by N^G-nitro-L-arginine methyl ester strongly indicate that the impairment of NOS activity may have caused dysfunction of the H_2S synthase/H_2S pathways leading to the increase in systemic blood pressure (Zhong *et al*., 2003). It has also been reported that in spontaneously hypertensive rats, H_2S generation is impaired and that exogenous H_2S might exert beneficial effects in the pathogenesis of spontaneous hypertension (Yan *et al*., 2004). In the light of these results it is reasonable to speculate that in the case of the aggravation of myocardial ischaemia–reperfusion injury by L-NMMA in the present experiments, H_2S generation in

cardiac endothelial cells may have been reduced. However, we have not yet fully determined CSE activity and H_2S formation in hearts deprived of NO and submitted to ischaemia–reperfusion, and current experiments should verify this critical point.

In conclusion, the present results indicate that the H_2S-releasing diclofenac derivative, in spite of a blockade of prostaglandin formation, has noteworthy anti-ischaemic activity in the reperfused rabbit heart. GSH generation and opening of the K_{ATP} appear to be part of its mode of action although other mechanisms cannot be ruled out. The previous observation that nitro-aspirin (Rossoni *et al*., 2000) and nitro-naproxen (Rossoni *et al*., 2004) have anti-ischaemic effects similar to *S*-diclofenac and NaHS further illustrates that gaseous molecules such as H_2S and NO, albeit with different mechanism(s) and suggestion of 'cross talk' (Moore *et al*., 2003) and synergy (Hosoki *et al*., 1997), provide some protection in cardiovascular pathology. The pharmacological profile of *S*-diclofenac and its documented anti-inflammatory activity, with reduced gastrointestinal side effects (Bhatia *et al*., 2005; Li *et al*., 2007; Wallace *et al*., 2007), open up the way to a range of therapeutic applications also in cardiovascular disease, for instance in the treatment of myocardial ischaemia and prevention of infarct progression.

Conflict of interest

Dr Piero Del Soldato is a shareholder of CTG Pharma, Milan, Italy. This company has patents on reagents used in this study. Professor Anna Sparatore received a grant from CTG Pharma.

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