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The effect of NCX4016 on the consequences of ischaemia and reperfusion in the streptozotocin diabetic rat

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Non-standard abbreviations

MABP – mean arterial blood pressure

VBP – ventricular premature beats

VF – ventricular fibrillation

VT – ventricular tachycardia

CAO – coronary artery ligation

Abstract

The aim of this study was to assess the effect of chronic administration of NCX4016, a nitric oxide-releasing aspirin derivative on the consequences of coronary artery occlusion in streptozotocin-diabetic rats. Rats were made diabetic by injection of streptozotocin (60 mg kg^{-1}) and received insulin (2.5 U kg^{-1} s.c.), daily for 4 weeks. Animals received (i) Vehicle (PEG_{400} 1 ml kg^{-1}), (ii) aspirin (65.2 mg kg^{-1}), (iii) NCX4016 (60 mg kg^{-1}), or (iv) NCX4016 (120 mg kg^{-1}) orally, once daily for the last 5 days before coronary artery occlusion (CAO). One hour following the last dose, pentobarbital-anaesthetised rats were subjected to CAO for 30-min followed by 120-min reperfusion. Neither drug significantly modified initial hemodynamics or plasma glucose levels compared to vehicle treatment in either non-diabetic or diabetic rats. Neither drug modified the total ventricular premature beats (VPB) count in normal animals, although NCX4016, but not aspirin, reduced the total VPB count and the incidence of ventricular tachycardia in diabetic rats. In non-diabetic animals, both aspirin and NCX4016 reduced infarct size. However, in diabetic rats infarct size was reduced only by the larger dose of NCX4016 (120 mg kg^{-1}) but not by aspirin or the lower dose of NCX4016. These results demonstrate that the cardioprotective effects of NCX4016 are reduced in the presence of diabetes compared to the effects seen in non-diabetic animals. In summary, the present study confirms the protective effect of NCX4016 against ischemia-reperfusion injury in the normal rat heart and demonstrates for the first time its protective effect in the heart of streptozotocin-diabetic rats.

Introduction

To date, aspirin is the most effective drug in the prevention of primary and secondary thrombotic events that lead to myocardial infarction (Mehta, 2002). This effect is attributed to its ability to inhibit platelet thromboxane production via cyclooxygenase inhibition (Vane, 1971). However, the use of aspirin is limited by the gastric ulceration that results from cyclooxygenase inhibition in the mucosa (Wolfe et al., 1999). Furthermore, there is conflicting experimental evidence regarding its ability to afford any direct cardioprotection against serious ventricular arrhythmias (Wainwright and Parratt, 1991) or myocardial injury (Schoemaker et al., 1998) in therapeutic doses. Thus, while aspirin may prevent an acute coronary event, it may not improve the outcome (Verheugt et al., 1990). Therefore a goal of any new compound would be the prevention of thrombotic occlusion with the ability to protect the heart should an ischaemic event occur.

NCX4016 (2 acetoxy-benzoate 2-(2-nitroxymethyl)–phenyl ester) is a novel ester which combines aspirin with a nitric oxide (NO) releasing moiety and was developed to improve the gastric tolerability of aspirin (Del Soldato et al., 1999). It is thought that the release of NO from the compound compensates for the loss of protective prostaglandins that result from cyclooxygenase inhibition. Experimental studies have confirmed that NCX4016 is devoid of any gastric toxicity in normal (Wallace et al., 1999) and diabetic (Tashima et al., 2000) rats. In addition, NCX4016 was shown to protect the gastric mucosa in a dose dependent manner (Takeuchi et al., 1998).

Like aspirin, NCX4016 was found to inhibit platelet aggregation *in vitro* (Minuz et al., 1995;Lechi et al., 1996b) and *in vivo* (Wallace et al., 1999) . Some reports have even suggested that NCX4016 may be a more potent inhibitor of platelet aggregation than aspirin and these effects may be related to the release of NO from the compound (Lechi et al.,

1996a). *In vivo* studies have shown NCX4016 to be cardioprotective in rabbits subjected to coronary artery ligation (Rossoni et al., 2000). Similarly, treatment of rats with NCX4016 for 5 days protected the heart from damage and arrhythmias induced by ischaemia and reperfusion (Rossoni et al., 2001), while a similar pre-treatment regime in pigs dose-dependently reduced infarct size and ventricular premature beats following myocardial ischemia and reperfusion (Wainwright et al., 2002). In all of these studies "native" aspirin failed to demonstrate a protective effect, despite clear evidence of inhibition of TxA_2 production. This implies that the cardioprotection afforded by NCX4016 is through a mechanism other than cyclooxygenase inhibition alone.

Diabetes mellitus is a major risk factor for cardiovascular disease, which accounts largely for the higher mortality and morbidity of diabetic populations (Vinik and Flemmer, 2002). The underlying mechanisms are a complex interplay of various factors including increased thrombotic potential (Kluft and Jesperson, 2002) and endothelial dysfunction through increased oxidative stress (Bayraktutan, 2002). NCX4016 has been shown to improve vascular endothelial function in streptozotocin-diabetic rats (Pieper et al., 2002), which suggests that this agent might improve the outcome of AMI in the diabetic state. However, while NCX4016 can protect the heart of normal animals, there is no data about the ability of NCX 4016 to protect the hearts of diabetic rats. Therefore the present study was undertaken to a) determine the effects of 5 days of treatment with aspirin or NCX4016 on infarct size and arrhythmias following coronary ligation and reperfusion in a rat model of diabetes mellitus compared with non-diabetic rats and b) to investigate the mechanism of the cardioprotective effect of NCX4016, the hypothesis being that the presence of a NO moiety would confer greater protection compared to the aspirin moiety alone.

Methods

Male Sprague-Dawley (SD) rats, weighing 300-400 g, were obtained from Harlan (Oxon, UK) and housed in the University of Strathclyde Biological Procedures Unit for 1 week of acclimatization prior to commencing the study. Animals were maintained at a temperature of 21 ± 2^{0}C, with a 12h light dark cycle and with free access to food and tap water. All studies were performed under an appropriate Project Licence authorised under the UK Animals (Scientific Procedures) Act 1986.

Induction of diabetes

Rats were given a single injection of streptozotocin (STZ, 60 mg kg^{-1}.*i.p.*; Sigma Aldrich, Poole, UK) to induce diabetes. Age and weight matched control rats (n=40) were injected with saline (1 ml kg^{-1}, *i.p.*). Any STZ injected animal that did not exhibit glycosuria (glucose reagent strips, Bayer plc, Berks, UK) after 72h was excluded from further study. The failure rate was 7.5%. Diabetic animals were given protamine zinc insulin (PZI; CP Pharmaceuticals, Wrexham, UK; 2.5 U kg^{-1}, *s.c.*) daily for 4 weeks. One animal was subsequently excluded due to excessive (>10%) weight loss. Thus the total number of diabetic rats to receive subsequent drug or vehicle treatment was 36. The time matched, non-diabetic control animals received saline (1 ml kg^{-1}, *s.c.*) for 4 weeks. The last injection of PZI was given approximately 24 hours prior to anaesthesia for the study of myocardial ischaemia and reperfusion. Water, food and body weights were monitored daily throughout the duration of the experiment.

Experimental protocol

For the last 5 days of the 4 week period rats were allocated to one of four treatment regimes. Animals were pre-treated orally, via a gavage tube advanced directly into the stomach, with polyethylene glycol (PEG_{400} 1 ml kg^{-1}; Sigma Aldrich, Poole, UK; n=10 diabetic, 10 non-diabetic), aspirin (ASA - 65.2 mg kg^{-1}; NICOX S.A., Nice, France; n=8 diabetic, 10-non-

diabetic), NCX4016 (60 mg kg^{-1}; NICOX S.A., Nice, France; n=9 diabetic, 10 non-diabetic) or NCX4016 (120 mg kg^{-1}; n=9 diabetic, 10 non-diabetic). Both NCX4016 and aspirin were dissolved in PEG_{400} prior to administration. The final dose of drug was given approximately one hour prior to anaesthesia. The dose of aspirin selected for the study is equi-molar to the high dose of NCX4016.

In vivo coronary artery occlusion

At the end of the pre-treatment protocol rats were anaesthetised with sodium pentobarbital (Sagatal®; Rhône Mérieux, Dublin, Ireland; 60 mg kg^{-1}, *i.p.*) followed by cannulation of the trachea and the left jugular vein. Arterial blood pressure was recorded via the left carotid artery using a pressure transducer (DTX Plus, Becton Dickinson, Ltd, UK) and the animal prepared for *in vivo* occlusion of left anterior descending coronary artery (Clark et al., 1980) through a left thoracotomy, with rats ventilated on room air (Harvard small animal respiration pump; 54 strokes per min; tidal volume 1.5 ml/100 g to maintain PCO_2 at 18-24 mmHg, PO_2 at 100-130 mmHg and pH at 7.4). The ECG was recorded using standard lead 1 subcutaneous electrodes. Data from the pressure transducer and the ECG leads were integrated using Po-Ne-Mah pre-amplifiers and the signals sent to a Powerlab data acquisition system (AD Instruments) for storage and analysis. Anaesthesia was maintained throughout by administration of sodium pentobarbitone (30 mg kg^{-1}) via the venous cannula every 30 minutes, or as required.

After placement of the ligature rats were allowed to stabilize for 15 minutes before the ligature was then tightened to induce regional ischaemia. Following 30 minutes of ischaemia, the ligature was loosened to restore blood flow to the myocardium. The heart was then reperfused for 2 hours. At the end of the experimental protocol approximately 5 ml of blood was withdrawn from the arterial cannula. Following withdrawal of blood rats were

euthanised by an overdose of anaesthetic and the hearts excised for analysis of area at risk and infarct size. The ventricular area at risk was determined by perfusing the heart via the aorta, after flushing out residual blood using saline (2ml) the ligature was re-tightened and 2ml of 0.5% w/v solution Evan's blue (Sigma Aldrich, Poole, UK) perfused (Sheehan and Epstein, 1983). Hearts were then immediately frozen at -20^{o}C for no longer than 12 hours. The frozen hearts were sliced from apex to base in 2-3mm sections and, after defrosting, the infarct was delineated by incubating sections at 37^{o}C for 15 min with 1% triphenyltetrazonium chloride (Sigma Aldrich, Poole, UK) (Vivaldi et al., 1985) in phosphate buffered saline. The sections were fixed overnight in formal saline and then photographed using a Nikon 1000 digital camera. Left ventricular area, area at risk (AAR) and infarct size (IS) were determined using computerised planimetry (Image-Pro Express, Media Cybernetics, USA). AAR was expressed as a percentage of total left ventricular area and IS was expressed as a percentage of the AAR.

Plasma analysis

Plasma was prepared from whole blood centrifuged (Scotlab Micro Centaur, UK) at 6500 rpm for 10 minutes. Plasma glucose was determined using a Beckman Glucose Analyzer (Beckman Instruments). Plasma salicylate was determined using HPLC, plasma nitrate was converted to nitrite by incubation with nitrate reductase and total nitrite concentration was determined by the Greiss reaction (Verdon et al., 1995) and plasma free thyroid hormone (FT_3 and FT_4) concentration was determined by radioimmunoassay (Zhang et al., 2002). To directly assess platelet cyclooxygenase function, whole blood was incubated with the calcium ionophore A23187 (Sigma Aldrich, Poole, UK; 30 μM) for 30 min at 37^{o}C, after which plasma was obtained by centrifugation (as described above). Plasma TxB_2 concentration was then determined by radioimmunoassay (Warner et al., 1999).

Statistical analysis

Data between two groups were compared using Student's t test. Data involving more than two groups were analysed using one way ANOVA with Tukey's *post hoc* test, statistical significance was accepted when $P<0.05$. Mean arterial blood pressure and heart rate was analysed in three sections; stabilisation, ischaemia, and reperfusion. Differences between drug treatment groups were analysed by 2 way ANOVA. Ventricular arrhythmias were determined from the ECG trace and classified using the Lambeth convention (see Wainwright et al., 2002). Arrhythmias are expressed as mean ± SEM and statistical difference was analysed by one way ANOVA with Tukey's post-hoc test. The incidence of severe arrhythmias is expressed as a percentage of the total number of animals in each group and the statistical differences between the groups were determined using Fisher's exact test.

Results

Exclusions

Of the 76 rats entered into the drug study, a total of 7 animals were excluded due either to technical problems or to animals developing arrhythmias during the stabilization period. These exclusions were spread evenly across all drug groups in both the diabetic and non-diabetic groups.

Streptozotocin induced diabetes

There was no difference in body weight at the start of the experimental period between normal (335 ± 5.5 g) and diabetic rats (340 ± 4.6 g). Over the 4-week experimental period control animals exhibited weight gain and the body weights were significantly higher at the end of the experimental period (414 ± 7.2 g, $P<0.001$). However the diabetic animals failed to gain weight over the experimental period and the body weight was significantly lower than control animals at the end of the experiment (344 ± 6.3 g, $P<0.001$). There was no significant

difference in body weight between any of the drug treatment groups in either normal or diabetic animals (data not shown).

Diabetic animals exhibited increased food (normal rats 193 ± 4, diabetic rats 287 ± 6 g per rat per week, $P<0.001$) and water intake (normal rats 210 ± 8, diabetic rats 704 ± 24 g per rat per week, $P<0.001$) during the experimental period. None of the drug treatments significantly modified food or water intake in the final week of the experiment in either normal or diabetic animals (data not shown). Diabetic rats had a significantly higher plasma glucose concentration compared to normal animals (normal 13 ± 1, diabetic 45 ± 3 mmol L^{-1}, $P<0.001$). None of the drug treatments modified plasma glucose concentrations in either normal or diabetic animals (data not shown). In addition, diabetic rats had significantly reduced free triiodothyronine (FT_3) (3.6 ± 0.2 pmol L^{-1}, $P<0.05$) and free thyroxine (FT_4) (25.6 ± 1.8 pmol L^{-1}, $P<0.05$) concentrations when compared to normal control rats (4.3 ± 0.17, 31.3 ± 1.2 pmol L^{-1}, respectively).

In vivo ischaemia and reperfusion

There was no difference in initial mean arterial blood pressure (MABP) between normal (111 ± 10 mmHg) and diabetic (105 ± 7 mmHg) control rats or in initial HR between normal (373 ± 17 bpm) and diabetic (378 ± 18 bpm) rats. None of the drug treatments modified MABP or HR from control values in normal or diabetic rats (data not shown). During stabilisation, ischaemia and reperfusion there was no significant difference in MABP (Table 1a) or HR (Table 1b) between normal and diabetic control (PEG_{400} treated) animals and both groups exhibited the characteristic drop in MABP upon occlusion of the coronary artery (Table 1a). However, normal rats had a significantly higher HR during ischaemia ($P=0.04$) and reperfusion ($P<0.0001$) compared to diabetic rats.

During stabilisation there was no significant difference in MABP between any of the drug groups in normal rats (Table 1a). During ischaemia, MABP was significantly lower in aspirin and high dose NCX4016 treated rats when compared to controls. During reperfusion, there was no difference between any group and controls, although low dose NCX4016 treated rats had a significantly higher MABP when compared to those receiving high dose NCX4016. Aspirin significantly reduced heart rate throughout the protocol in normal rats when compared to controls (Table 1b). None of the other drug treatments had any significant effect on HR during stabilisation, ischaemia or reperfusion.

In diabetic animals, aspirin significantly reduced MABP when compared to diabetic controls during stabilisation (Table 1a). During ischaemia animals receiving NCX4016 at either dose or aspirin all demonstrated a significantly reduced MABP compared to control rats. However, during reperfusion only low dose NCX4016 significantly reduced MABP with respect to controls. MABP was also significantly lower in the low dose NCX4016 group when compared to rats pre-treated with high dose NCX4016. As in normal rats, aspirin significantly reduced HR throughout the ischaemia and reperfusion protocol when compared to all groups. High dose NCX4016 significantly reduced HR during ischaemia, while low dose NCX4016 only reduced HR during reperfusion (Table 1b).

There was no significant difference in the total number of arrhythmias that occurred during ischaemia between normal and diabetic rats (Figure 1). There was also no difference between normal and diabetic animals in the time course of arrhythmic activity, with the majority of arrhythmia occurring between 6 and 15 minutes of ischaemia in both normal and diabetic rats, or in the incidence of severe arrhythmias (VT, VF; Figure 2). Neither aspirin nor NCX4016, at either, dose had any significant effects on the development of arrhythmias in normal rats. However, while aspirin had no effect on the number of arrhythmias in the diabetic rats, the lower dose of NCX4016 showed a marked tendency to reduce the total

arrhythmia count and at the higher dose NCX4016 significantly reduced ventricular ectopic activity ($P<0.05$) (Figure 1). None of the drug treatments significantly modified the incidences of VT or VF in normal rats, although NCX4016 reduced the incidence of VT ($P<0.05$) in diabetic rats (Figure 2b). Furthermore, there was no difference in the total number or the incidences of severe reperfusion arrhythmias between normal and diabetic animals and none of the drug treatments had any effect (Data not shown).

There were no significant differences in either the area at risk (AAR) (normal 57 ± 3%, diabetic 59 ± 4%) or infarcted area between normal (41 ± 4%) and diabetic rats (41 ± 6%). AAR was not different in any of the drug treatment groups in the normal or diabetic rats (Figure 3). Aspirin and the low dose of NCX4016 significantly reduced infarct size when compared to controls, although the reduction in infarct size observed with high dose NCX4016 failed to reach statistical significance ($P=0.08$). In diabetic animals the AAR was also similar in all drug treatment groups. However, while neither aspirin nor the low dose of NCX4016 had any effect, the high dose of NCX4016 significantly reduced infarct size when compared to controls.

Biochemical analysis

There was no difference in circulating TxB_2 concentrations between normal and diabetic control rats (3.6 ± 2.4, 3.3 ± 2.6 ng ml^{-1}, respectively). *Ex vivo* activation of platelets with calcium ionophore (30 μM A23187) caused a similar increase in platelet TxB_2 production in blood from both normal (26.7 ± 3.4 ng ml^{-1}) and diabetic (20.7 ± 4 ng ml^{-1}) rats (Figure 4). None of the drug treatments had any effect on circulating TxB_2 levels in normal rats compared to controls. However, aspirin significantly reduced TxB_2 production in response to *ex vivo* platelet activation with A23187 compared to controls, whereas NCX4016 had no effect on TxB_2 production at either dose (Figure 4a). In the diabetic rats none of the drug

treatments had any effect on circulating TxB_2 levels compared to controls. Similar to the findings in blood from normal rats, aspirin significantly reduced TxB_2 production in response to *ex vivo* activation with A23187 compared to controls, while NCX4016 had no significant effect on TxB_2 production at either dose (Figure 4b).

In normal rats the plasma concentration of the aspirin breakdown product, salicylate, achieved in rats pre-treated with high dose NCX4016 (33.9 ± 7.2 μg ml^{-1}) was approximately 50% of that achieved in rats treated with an equimolar dose of aspirin (78.7 ± 11.2 μg ml^{-1}; $P<0.001$). In addition, the salicylate concentration achieved in rats pre-treated with low dose NCX4016 (18.0 ± 4.8 μg ml^{-1}) was approximately 50% the level achieved with the higher dose of NCX4016, although the difference was not significant ($P=0.0795$). Diabetic rats had slightly lower plasma salicylate concentrations in each treatment group when compared to normal rats, although there was no statistical difference between the two groups. As in the normal rats, the high dose of NCX4016 produced a significantly lower plasma salicylate concentration compared to an equimolar dose of aspirin (18.8 ±3.9 vs 51.9 ± 0.7 μg ml^{-1} respectively). The concentration of salicylate achieved in the lower dose group of NCX4016 (16.2 ± 2.3 μg ml^{-1}) was also significantly lower than in the aspirin group, but not significantly different from the higher dose NCX4016 group.

There was no significant difference in plasma NO_x (NO_2 + NO_3) concentrations between normal and diabetic control rats (Table 2). Five days of pre-treatment with aspirin or the lower dose of NCX4016 had no significant effect in normal but the higher dose of NCX4016 significantly increased NO_x concentration. However, in the diabetic rats there was no difference in NO_x concentration in any of the drug treatment groups (Table 2).

Discussion

Although clinical studies have shown that the diabetic heart is more susceptible to ischemic injury, the effects of experimental diabetes remains unclear (Paulson, 1997). Several studies have shown that the diabetic rat heart is less susceptible to arrhythmias *ex vivo* and *in vivo* (Ravingerova et al., 2001). However, this effect may be explained by the hypothyroidism that accompanies streptozotocin diabetes, as it was abolished by thyroid replacement therapy or by insulin in a dose that normalised blood glucose and prevented hypothyroidism (Zhang et al., 2002). In the present study, administration of insulin was primarily intended to prevent streptozotocin diabetes-induced hypothyroidism, while maintaining marked symptoms of diabetes**.** This was achieved, as evidenced by the persistence of marked hyperglycaemia, reduced weight gain, polyuria, glycosuria, polyphagia, polydypsia while seeing only small decreases in plasma free thyroid hormone concentrations compared to those reported in untreated diabetic rats (Zhang et al. 2002). The high plasma glucose levels may reflect the time (24h) between the last dose of insulin and measurement. In pilot studies when insulin was given 1 hour before anaesthesia, the rats were euglycemic (i.e plasma glucose of 10 mmol L^{-1}). The mild reduction in thyroid hormones is consistent with the absence of differences between the resting heart rates of control and STZ-diabetic rats. It thus appears that, in the absence of clinical hypothyroidism, STZ-diabetes of 4-week duration and poorly controlled by insulin, is without significant effect on arrhythmias or infarct size following ischaemia and reperfusion. Moreover, STZ-diabetes had no effect on platelet activity, as evidenced by the absence of any difference between non-diabetic and diabetic rats in the generation of TxB_2 in response to A23187. Several studies have shown that platelets from diabetic animals are bigger (Judge et al., 1995) and produce more thromboxane B_2 when activated (De La et al., 2002) than platelets from normal animals. The insulin regimen used here may have been adequate to prevent platelet hyperactivity.

There were, however, clear differences between the effects of the drugs in normal and in diabetic rats. Neither aspirin nor NCX4016 influenced ischaemia-induced arrhythmias in normal rats, whereas the higher dose of NCX4016, but not aspirin, reduced the number of ventricular ectopic beats following ischaemia in the diabetic rats. Furthermore, in normal rats, both aspirin and NCX4016 reduced infarct size, whereas in diabetic rats only NCX4016 was effective in this respect. The overall protective effect of NCX4016 is consistent with previous findings (Rossoni et al. 2001), although these authors showed ischaemia-reperfusion cardiac arrhythmias to be reduced by both NCX4016 and aspirin in normal rats. These observations raise questions concerning the mechanism whereby NCX4016 exerts it protective effect and the greater effectiveness of NCX4016 compared with aspirin in this model of diabetes. As NCX4016 did not modify plasma glucose concentrations, its effectiveness in diabetic animals was not due to modification of the diabetic state.

NCX4016 is rapidly metabolised *in vitro* (within 5 min) to salicylic acid and 3-(nitrooxymethyl)phenol (Carini et al., 2002), the latter resulting in increases in plasma nitric oxide, as evidenced by an increase in plasma nitrosylhemoglobin and nitrate/nitrite concentrations (Carini et al., 2001). Plasma salicylate concentrations provide a good surrogate marker for NCX4016 (Carini et al., 2004) and probably contribute, along with nitric oxide, to the pharmacology of NCX4016. However, other metabolites of NCX4014 or the intact compound itself may contribute to the pharmacological profile. In this context, NCX4016 was recently shown to directly inhibit cyclooxygenase-1 *in vitro* (Corrazzi et al. 2005). The present data suggest that the effects of NCX4016 on infarct size or arrhythmias (in diabetic rats) are unlikely to be due solely to the aspirin moiety. First, the profile of protection seen with NCX4016 is clearly different from that of aspirin in the diabetic rat, with NCX4016, but not aspirin, reducing both arrhythmias and infarct size. These effects of NCX4016 were

evident despite the markedly lower plasma salicylate concentration following administration of NCX4016 compared with that achieved following an equimolar dose of aspirin. Second, aspirin, but not NCX4016, reduced thromboxane B_2 generation from blood samples in response to the calcium ionophore A23187. Some studies have similarly reported a lack of effect of NCX4016 on TxB_2 production by platelets in rats (Bak et al., 1998), whereas in other species platelet TxB_2 production was inhibited by NCX4016 (Wainwright et al., 2002). Furthermore, NCX4016 does appear to be an effective antithrombotic agent in *in vivo* models of thrombus formation (Wallace et al., 1999). We did not measure platelet aggregation in this study but our observations in spontaneously hypertensive rats have shown that NCX4016 (120 mg kg^{-1} daily for five days), in contrast to aspirin, does not modify collagen-induced platelet aggregation *ex vivo*. In that model NCX4016 inhibited aggregation only when administered twice daily in the same dose (unpublished observations). While we cannot rule out an effect on local platelet activation *in vivo* over the time course of our experiments, the beneficial effect of NCX4016 against arrhythmias and myocardial injury in the present study is unlikely to be simply related to an anti-platelet effect.

The cardioprotective action of NCX4016 may be due to the release of the nitric oxide moiety, since there is considerable evidence that both endogenous nitric oxide production (Jones et al., 2004) and nitric oxide donors (Pernow and Wang; 1999) are protective against myocardial ischaemia-reperfusion injury. However, we could not demonstrate a direct correlation between plasma NOx and cardioprotection, since increased plasma nitrate/nitrite concentrations were only observed in non-diabetic rats following administration of NCX4016 in the larger dose and could not be demonstrated in diabetic rats. Carini et al., (2001) showed that the time-course of increases in plasma nitrate/nitrite concentrations was similar to that of increases in nitrosylhemoglobin, a much more sensitive indicator of bioactive nitric oxide,

and peaks around 4-6 hours after oral administration. Therefore**,** the failure to detect increases in plasma nitrate/nitrite with the lower dose of NCX4016 or with the higher dose in diabetic rats does not refute a role for nitric oxide, as the concentrations were measured at only one time (3.75h) after the last oral dose**.** Further, NO released from NCX4016 can also be stored as nitrosylhemoglobin and S-nitrosothiols following oral dosing (Carini et al., 2004). Since NO can be transported in forms that would not be detected by the assay employed here, it is likely that measurement of NO*x* is underestimating the amount of bioavailable NO. Comparison with an equivalent dose of a conventional nitric oxide donor, while potentially informative, would be complicated by the profound hypotension produced by these agents, in contrast to the minimal effect of NCX4016, which releases NO only very slowly (Keeble et al 2001).

An alternative explanation for the beneficial effects seen with NCX4016 may be due to an interaction between salicylate and nitric oxide. A synergistic effect between aspirin and nitric oxide (formed from L-arginine) on the recovery of the heart from ischaemia has been demonstrated previously in rat isolated perfused hearts subjected to global ischaemia followed by reperfusion (Wanna et al., 1995). Recent work has also shown that the effects of NCX4016 on neutrophil/endothelium interactions are mediated by both the aspirin moiety and nitric oxide, with nitric oxide being more important (Fiorucci et al., 2004).

The reduction in heart rate seen at various stages during ischaemia and reperfusion with aspirin and NCX4016 may contribute to their beneficial effects by reducing oxygen demand; aspirin reduced heart rate throughout ischaemia and reperfusion in both diabetic and control rats, while NCX4016 reduced heart rate only during reperfusion with the high dose and only during ischaemia for the low dose. An earlier study in conscious, chronically infarcted rats

showed aspirin to reduce post-infarction heart rate, while maintaining cardiac output (Schoemaker et al., 1998). Since we did not measure cardiac output we cannot speculate on any effect of the agents on this determinant of oxygen demand. However, both drugs reduced mean arterial blood pressure during ischaemia and the larger dose of NCX4016 reduced blood pressure during reperfusion in the diabetic rats, thereby reducing afterload.

The failure of aspirin to exert any protection in diabetic rats has no immediate explanation. Aspirin appeared equally effective in normal and diabetic rats in inhibiting the calcium ionophore-induced thromboxane production *ex vivo*. Perhaps in diabetic rats there is a greater dependency upon endogenous cardioprotective prostanoids generated during ischaemia (Wainwright & Parratt 1991); aspirin may remove this endogenous cardioprotective mechanism. This may be consistent with the requirement for a higher dose of NCX4016 to afford protection in the diabetic hearts, indicating a need for larger amounts of NO to overcome the salicylate-induced removal of the endogenous prostanoid protective mechanism.

In summary, the present study confirms the protective effect of NCX4016 against ischaemia-reperfusion injury in the normal rat heart and demonstrates for the first time its protective effect in the heart of streptozotocin-diabetic rats. NCX4016 may protect the diabetic heart through a mechanism dependent upon the NO rather than the salicylate moiety of the molecule. Furthermore the higher dose of NCX4016 needed to exert this protection suggests that diabetic patients may require modified dosages compared to non-diabetic patients.

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Footnotes

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Figure Legends

Figure 1: Total number of arrhythmias observed during ischaemia in normal and diabetic rats pre-treated with either PEG_{400} (normal n=6; diabetic n=7), Aspirin (normal n=6; diabetic n=5), low dose NCX4016 (normal n=7; diabetic n=5) or high dose NCX4016 (normal n=8; diabetic n=7). **P*<0.05 compared to corresponding control.

Figure 2: Incidence of severe arrhythmias (expressed as a percentage of total number of animals in each treatment group) during ischaemia in normal (a) and diabetic (b) rats pre-treated with either PEG_{400} (normal n=10; diabetic n=9), Aspirin (normal n=9; diabetic n=7), low dose NCX4016 (normal n=8; diabetic n=8) or high dose NCX4016 (normal n=9; diabetic n=8). **P*<0.05 compared to control value. Statistical analysis was undertaken using Fisher's ExactTest.

Figure 3: Area at risk (AAR) and infarct size (IS) following ischaemia and reperfusion protocol in normal (a) and diabetic (b) pretreated with either PEG_{400} (normal n=6; diabetic n=7), Aspirin (normal n=6; diabetic n=3), low dose NCX4016 (normal n=6; diabetic n=4) or high dose NCX4016 (normal n=6; diabetic n=5). AAR is expressed as a percentage of left ventricular area and IS expressed as percentage of AAR. **P*<0.05; $^{\dagger}P$=0.08 compared to control value.

Figure 4: TxB_2 concentrations measured in plasma samples obtained from normal (A) and diabetic (B) rats pre-treated with either PEG_{400} (normal n=9; diabetic n=9), Aspirin (normal n=9; diabetic n=7), low dose NCX4016 (normal n=8; diabetic n=6) or high dose NCX4016

(normal n=9; diabetic n=8). Basal samples were obtained from rats. Active samples were obtained from whole blood samples incubated with 30μM A23187 for 30 minutes. *P<0.05 compared to control value.

Table 1: The effects of aspirin and NCX4016 on a) MABP and b) HR in diabetic and non-diabetic rats subjected to myocardial ischemia and reperfusion. Statistical significance was determined by 2 way ANOVA comparing drug treatments during the three sections of the protocol i.e. -15-0 min stabilisation, 1-30min ischemia, 31-180min reperfusion.

a)

MABP (mmHg)	Non-diabetic				Diabetic			
Time (min)	Vehicle	Aspirin	NCX4016 60 $mgkg^{-1}$	NCX4016 120 $mgkg^{-1}$	Vehicle	Aspirin	NCX4016 60 $mgkg^{-1}$	NCX4016 120 $mgkg^{-1}$
-15	89 ± 10	92 ± 8	94 ± 6	79 ± 6	81 ± 5	83 ± 5	81 ± 7	77 ± 6
0	87 ± 10	83 ± 10	85 ± 11	75± 6	79 ± 4	71 ± 4**	84 ± 8	78± 6
1	53 ± 8	52 ± 3*	53 ± 6	47 ± 2**	61 ± 6	52 ± 5**	50 ± 5**	56 ± 3*
15	74 ± 13	53 ± 10*	55 ± 13	59 ± 5**	79 ± 10	59 ± 9**	56 ± 14**	67 ± 5*
30	71± 9	64 ± 11*	64 ± 16	56 ± 3**	79 ± 10	59 ± 14**	60 ± 6**	64± 6*
31	63 ± 3	54 ± 7	60± 7	59 ± 5	68 ± 8	62 ± 9	50 ± 3**	62 ± 4$^{\#}$
60	61 ± 6	74 ± 10	74 ± 13	57 ± 8$^{\#}$	64 ± 8	43 ± 8	48 ± 5**	68 ± 7$^{\#}$
120	65 ± 7	79± 16	90 ± 14	53 ± 5$^{\#}$	53 ± 4	45 ± 10	46 ± 7**	63 ± 6$^{\#}$
180	66 ± 17	67 ± 8	70 ± 8	59 ± 10$^{\#}$	59 ± 6	65 ± 8	49 ± 5**	48 ± 7

b)

HR (bpm)	Non-diabetic				Diabetic			
Time (min)	Vehicle	Aspirin	NCX4016 60 $mgkg^{-1}$	NCX4016 120 $mgkg^{-1}$	Vehicle	Aspirin	NCX4016 60 $mgkg^{-1}$	NCX4016 120 $mgkg^{-1}$
-15	339 ± 21	393 ± 25	401 ± 24	391 ± 630	365 ± 24	351 ± 17	381 ± 11	356 ± 616
0	429 ± 13	390 ±20**	434 ± 18	427 ± 11	405 ± 12	340 ± 7*	381 ± 19	380 ± 20
1	415 ± 12	398 ±18**	430 ± 16	429 ± 11	417 ± 9	346 ± 17*	407 ± 16*	395 ± 16
15	460 ± 15	409 ±18**	414 ± 18	419 ± 10	417 ± 19†	348 ± 5*	376 ± 14*	393 ± 20
30	438± 16	379 ±19**	421 ± 16	425 ± 9	409 ± 10†	354 ± 10*	357 ± 11*	382 ± 15
31	426 ± 18	376 ±23**	415± 14	424 ± 9	401 ± 13†	355 ± 6*	334 ± 15	378 ± 18*
60	427 ± 15	397 ±21**	412 ± 16	416 ± 14	285 ± 13††	327 ± 82*	351 ± 12	384 ± 12*
120	448 ± 8	391±32**	404 ± 13	403 ± 17	374 ± 8††	344 ± 10*	361 ± 11	386 ± 13*
180	442 ± 23	378 ±12**	385 ± 815	379 ± 20	401 ± 3††	353 ± 8*	370 ± 5	340 ± 20*

*P<0.05; **P<0.01 Compared to same time point in corresponding vehicle group. $^{\#}$P<0.05 compared to corresponding group of rats given NCX4016 60 mg kg^{-1}.

†P<0.05; ††P<0.01 compared to non-diabetic controls

	Vehicle	Aspirin	NCX4016 60 mg kg^{-1}	NCX4016 120 mg kg^{-1}
Non-Diabetic	23.7 ± 2.4	30.4 ± 5.0	23.7 ± 3.8	37.4 ± 4.9*
Diabetic	29.1 ± 3.3	24.5 ± 2.3	35.0 ± 5.7	25.4 ± 6.2

Table 2: Plasma NOx (NO_2 + NO_3) concentrations (μg ml^{-1}) in plasma from normal and diabetic rats pre-treated with either PEG_{400} (normal n=9; diabetic n=8), Aspirin (normal n=9; diabetic n=6), low dose NCX4016 (normal n=8; diabetic n=8) or high dose NCX4016 (normal n=9; diabetic n=8). Values are expressed as mean ± sem. *P<0.05 compared to control value.

Figure 1

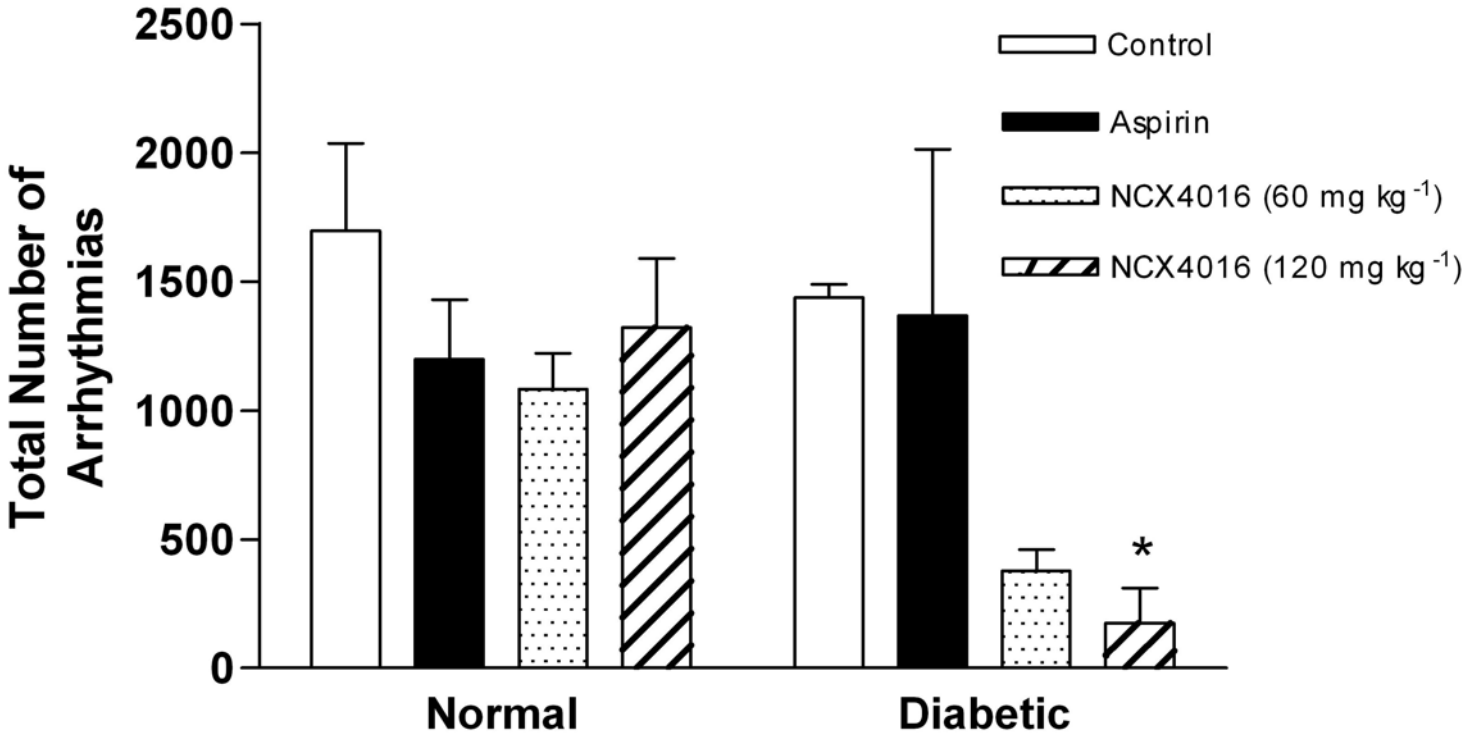
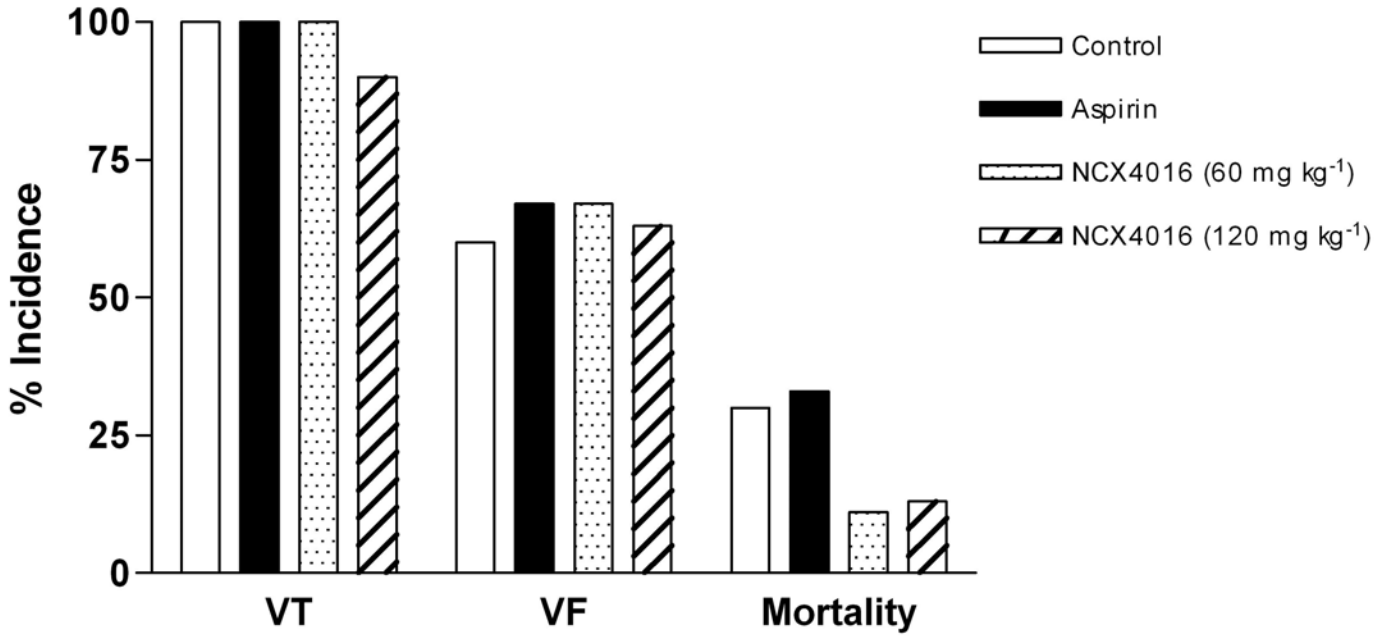

Total Number of Arrhythmias
2500
2000
1500
1000
500
0
Normal
Diabetic
*
Control
Aspirin
NCX4016 (60 mg kg -1)
NCX4016 (120 mg kg -1)

Figure 2

A


100
75
50
25
0
% Incidence
VT
VF
Mortality
Control
Aspirin
NCX4016 (60 mg kg-1)
NCX4016 (120 mg kg-1)

B

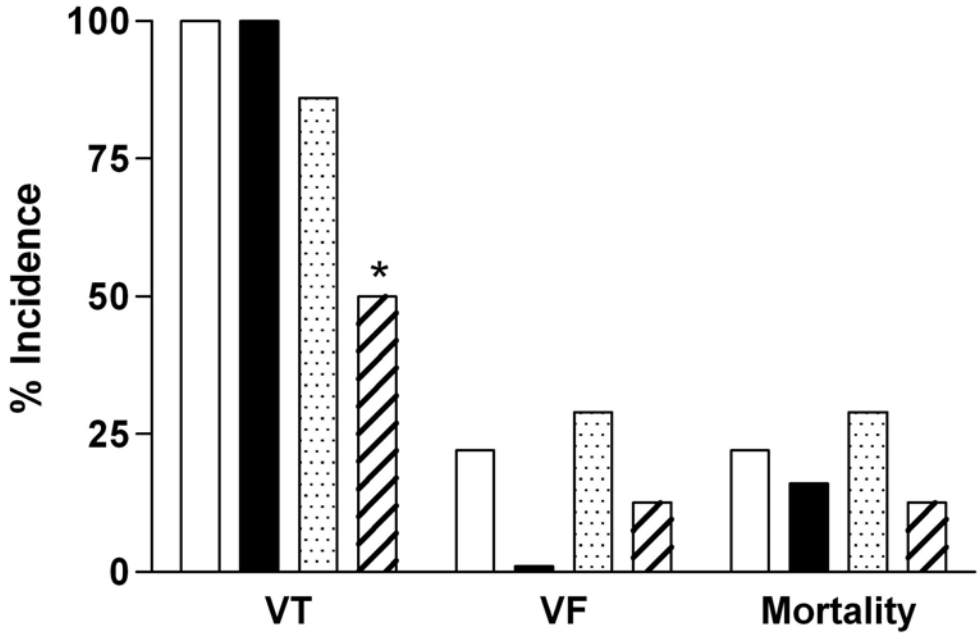
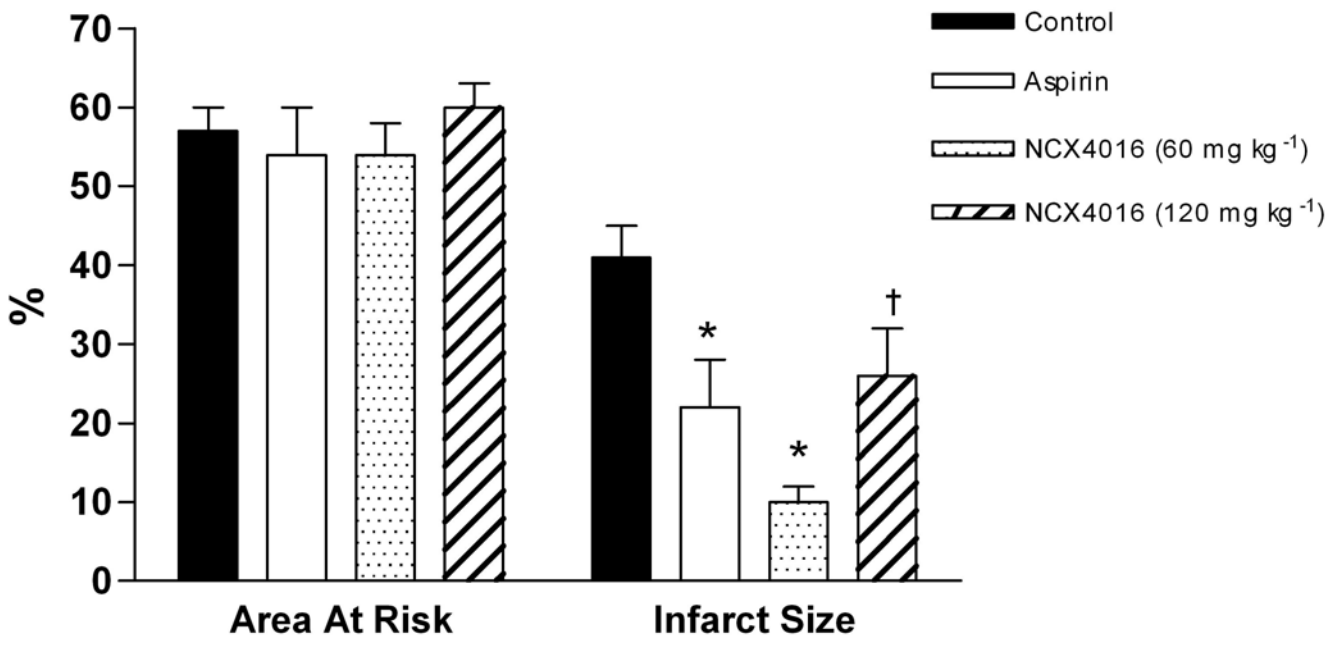

100
75
50
25
0
% Incidence
*
VT
VF
Mortality

Figure 3

A


70
60
50
40
30
20
10
0
%
Control
Aspirin
NCX4016 (60 mg kg -1)
NCX4016 (120 mg kg -1)
*
*
†
Area At Risk
Infarct Size

B

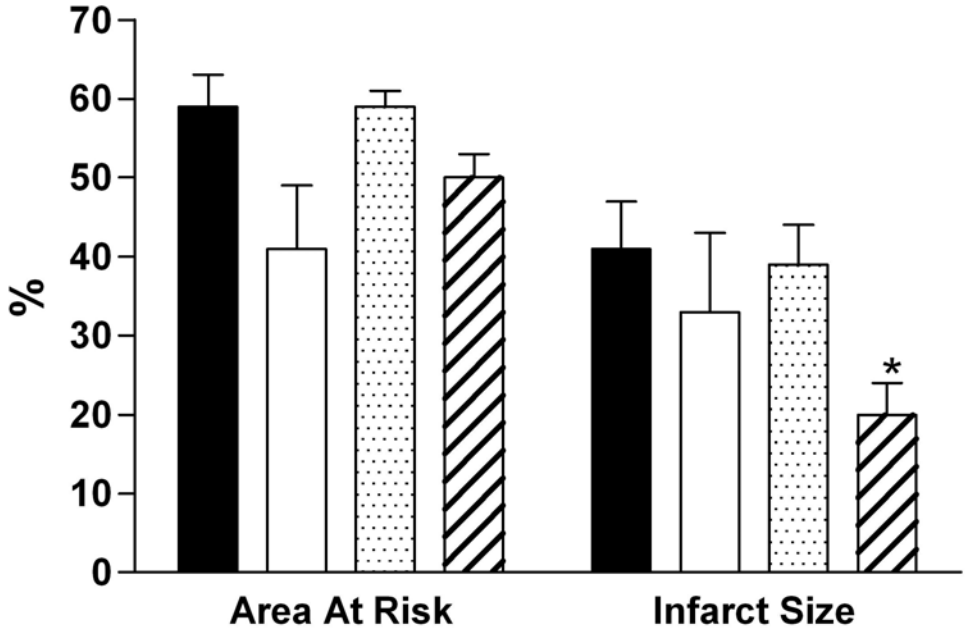
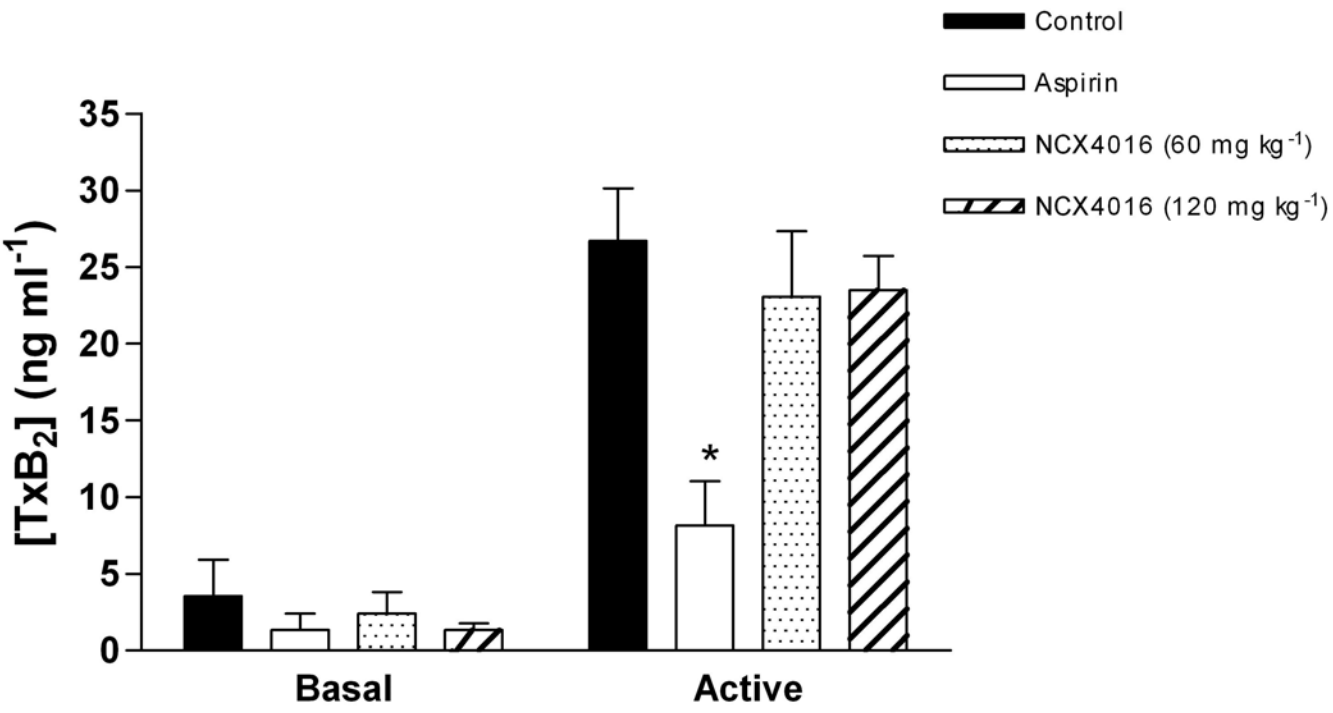
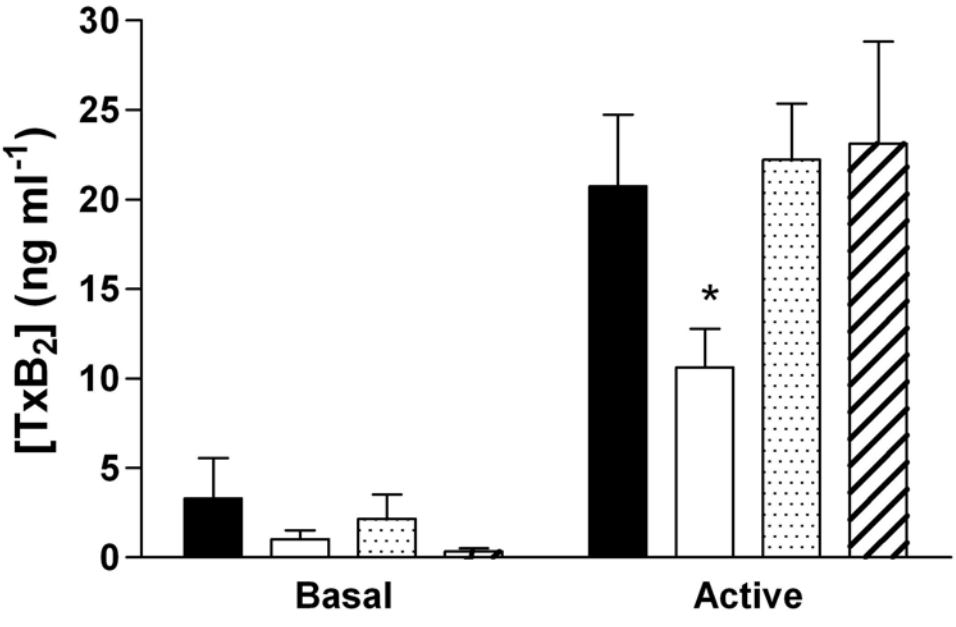

70
60
50
40
30
20
10
0
%
*
Area At Risk
Infarct Size

Figure 4

A


Control
Aspirin
NCX4016 (60 mg kg-1)
NCX4016 (120 mg kg-1)
[TxB2] (ng ml-1)
35
30
25
20
15
10
5
0
*
Basal
Active

B


[TxB2] (ng ml-1)
30
25
20
15
10
5
0
*
Basal
Active