The Effect of NCX4016 [2-Acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl Ester] on the Consequences of Ischemia and Reperfusion in the Streptozotocin Diabetic Rat

S. G. Burke, C. L. Wainwright, I. Vojnovic, T. Warner, D. G. Watson, and B. L. Furman

Departments of Physiology and Pharmacology (S.G.B., B.L.F.) and Pharmaceutical Sciences (D.G.W.), Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom; School of Pharmacy, Robert Gordon University, Aberdeen, United Kingdom (C.L.W.); and William Harvey Research Institute, Barts and the London, Queen Mary School of Medicine and Dentistry, London, United Kingdom (I.V., T.W.)

Received September 27, 2005; accepted October 28, 2005

ABSTRACT

The aim of this study was to assess the effect of chronic administration of NCX4016 [2-acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester], 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 vehicle (1 ml kg\(^{-1}\) polyethylene glycol), aspirin (65.2 mg kg\(^{-1}\)), 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 after the last dose, pentobarbital-anesthetized rats were subjected to CAO for 30 min followed by 120-min reperfusion. Neither drug significantly modified initial hemodynamics or plasma glucose levels compared with vehicle treatment in either nondiabetic or diabetic rats. Neither drug modified the total ventricular premature beat (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 nondiabetic 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 with the effects seen in nondiabetic 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.

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, although aspirin may prevent an acute coronary event, it may not improve the outcome (Vergeht 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 ischemic event occur.

NCX4016 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 its ability to inhibit platelet thromboxane production via cyclooxygenase inhibition in the mucosa (Wolfe 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).

S.G.B. was funded by a studentship from NICOX S.A. (Nice, France). Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.105.096539.

ABBREVIATIONS: NCX4016, 2-acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester; NO, nitric oxide; STZ, streptozotocin; PEG, polyethylene glycol; AAR, area at risk; A23187, calcimycin; TxB\(_2\), thromboxane B\(_2\); ANOVA, analysis of variance; MABP, mean arterial blood pressure; HR, heart rate; VT, ventricular tachycardia.
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). Likewise, treatment of rats with NCX4016 for 5 days protected the heart from damage and arrhythmias induced by ischemia and reperfusion (Rossoni et al., 2001), whereas a similar pretreatment regime in pigs dose-dependently reduced infarct size and ventricular premature beats after 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 TxA2 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, although NCX4016 can protect the heart of normal animals, there are no data about the ability of NCX 4016 to protect the hearts of diabetic rats. Therefore, the present study was undertaken to determine the effects of 5 days of treatment with aspirin or NCX4016 on infarct size and arrhythmias after coronary ligation and reperfusion in a rat model of diabetes mellitus compared with nondiabetic rats and 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 with the aspirin moiety alone.

Materials and Methods

Male Sprague-Dawley rats, weighing 300 to 400 g, were obtained from Harlan UK Limited (Bicester, Oxon, UK) and housed in the University of Strathclyde Biological Procedures Unit for 1 week of acclimatization before commencing the study. Animals were maintained at a temperature of 21 ± 2°C, with a 12-h light/dark cycle and with free access to food and tap water. All studies were performed under an appropriate Project License authorized 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 Chemical, Poole, Dorset, 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 AG, Wuppertal, Germany) after 72 h was excluded from further study. The failure rate was 7.5%. Diabetic animals were given protamine zinc insulin (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, nondiabetic control animals received saline (1 ml kg⁻¹ s.c.) for 4 weeks. The last injection of protamine zinc insulin was given approximately 24 h before anesthesia for the study of myocardial ischemia 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 pretreated orally via a gavage tube advanced directly into the stomach, with polyethylene glycol (1 ml kg⁻¹ PEG₄₀₀; Sigma Chemical; n = 10 diabetic, 10 nondiabetic), aspirin (65.2 mg kg⁻¹; NICOX S.A., Nice, France; n = 8 diabetic, 10 nondiabetic), NCX4016 (60 mg kg⁻¹; NICOX S.A.; n = 9 diabetic, 10 nondiabetic), or NCX4016 (120 mg kg⁻¹; n = 9 diabetic, 10 nondiabetic). Both NCX4016 and aspirin were dissolved in PEG₄₀₀ before administration. The final dose of drug was given approximately 1 h before anesthesia. The dose of aspirin selected for the study is equimolar to the high dose of NCX4016.

In Vivo Coronary Artery Occlusion. At the end of the pretreatment protocol, rats were anesthetized with sodium pentobarbital (Sagatal; Rhône Mérieux, Dublin, UK; 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 (Dxx Plus; BD Biosciences, San Jose, CA), and the animal was prepared for in vivo occlusion of the 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/min; tidal volume, 1.5 ml/100 g to maintain PCO₂ at 18–24 mm Hg, PO₂ at 100–130 mm Hg, and pH at 7.4). The ECG was recorded using standard lead 1 s.c. electrodes. Data from the pressure transducer and the ECG leads were integrated using Pne-Mah preamplifiers, and the signals were sent to a Powerlab data acquisition system (ADIInstruments Pty Ltd., Castle Hill, New South Wales, Australia) for storage and analysis. Anesthesia was maintained throughout by administration of sodium pentobarbital (30 mg kg⁻¹ i.v.) via the venous cannula every 30 min or as required.

After placement of the ligature rats were allowed to stabilize for 15 min before the ligature was then tightened to induce regional ischemia. After 30 min of ischemia, the ligature was loosened to restore blood flow to the myocardium. The heart was then reperfused for 2 h. At the end of the experimental protocol, approximately 5 ml of blood was withdrawn from the arterial cannula. After withdrawal of blood, rats were euthanized by an overdose of anesthetic, and the hearts were 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 (2 ml); the ligature was repositioned, and 2 ml of 0.5% w/v solution Evans blue (Sigma Chemical) was perfused (Sheehan and Epstein, 1983). Hearts were then immediately frozen at −20°C for no longer than 12 h. The frozen hearts were sliced from apex to base in 2- to 3-mm sections, and, after defrosting, the infarct was delineated by incubating sections at 37°C for 15 min with 1% triphenyltetrazonium chloride (Sigma Chemical) (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 (Nikon, Tokyo, Japan). Left ventricular area, area at risk (AAR), and infarct size (IS) were determined using computerized planimetry (Image-Pro Express; Media Cybernetics, Inc., Silver Spring, MD). 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 min. Plasma glucose was determined using a Beckman Glucose Analyzer (Beckman Coulter, Fullerton, CA). Plasma salicylate was determined using high-performance liquid chromatography, plasma nitrate was converted to nitrite by incubation with nitrate reductase, total nitrite concentration was determined by the Greiss reaction (Verdon et al., 1995), and plasma free thyroid hormone (free triiodothyronine and free thyroxine) concentration was determined by radioimmunoassay (Zhang et al., 2002). To directly assess platelet cyclooxygenase function, whole blood was incubated with the calcium ionophore A23187 (calcimycin; Sigma Chemical; 30 μM) for 30 min at 37°C, after which plasma was obtained by centrifugation (as described above). Plasma TxB₂ 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 analyzed using one-way ANOVA with Tukey’s post hoc test, and statistical significance was accepted when \( P < 0.05 \). Mean arterial blood pressure and heart rate were analyzed in three sections; stabilization, ischemia, and reperfusion. Differences between drug treatment groups were analyzed by two-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 ± S.E.M., and statistical difference was analyzed 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 seven 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 nondiabetic 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 (340 ± 4.6 g) rats. 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 g/rat/week; diabetic rats, 287 ± 6 g/rat/week; \( P < 0.001 \)) and water intake (normal rats, 210 ± 8 g/rat/week; diabetic rats, 704 ± 24 g/rat/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 with normal animals (normal, 13 ± 1 mmol l⁻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 (3.6 ± 0.2 pmol l⁻¹, \( P < 0.05 \)) and free thyroxine (25.6 ± 1.8 pmol l⁻¹, \( P < 0.05 \)) concentrations compared with normal control rats (4.3 ± 0.17 and 31.3 ± 1.2 pmol l⁻¹, respectively).

**In Vivo Ischemia and Reperfusion.** There was no difference in initial mean arterial blood pressure (MABP) between normal (111 ± 10 mm Hg) and diabetic (105 ± 7 mm Hg) 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 stabilization, ischemia, and reperfusion there was no significant difference in MABP (Table 1) or HR (Table 1) between normal and diabetic control (PEGintestinal-treated) animals, and both groups exhibited the characteristic drop in MABP upon occlusion of the coronary artery (Table 1). However, normal rats had a significantly higher HR during ischemia (\( P = 0.04 \)) and reperfusion (\( P < 0.0001 \)) compared with diabetic rats.

During stabilization, there was no significant difference in MABP between any of the drug groups in normal rats (Table 1). During ischemia, MABP was significantly lower in aspirin and high-dose NCX4016-treated rats compared with control.

**Table 1**
The effects of aspirin and NCX4016 on MABP and HR in diabetic and nondiabetic rats subjected to myocardial ischemia and reperfusion.

<table>
<thead>
<tr>
<th>Time</th>
<th>Nondiabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Aspirin 60 mg kg⁻¹</td>
</tr>
<tr>
<td></td>
<td>NCX4016</td>
<td>NCX4016</td>
</tr>
<tr>
<td>MABP  (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>81 ± 5</td>
<td>83 ± 5</td>
</tr>
<tr>
<td>−15</td>
<td>89 ± 10</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>0</td>
<td>87 ± 10</td>
<td>83 ± 10</td>
</tr>
<tr>
<td>1</td>
<td>53 ± 8</td>
<td>52 ± 3*</td>
</tr>
<tr>
<td>15</td>
<td>74 ± 13</td>
<td>53 ± 10*</td>
</tr>
<tr>
<td>30</td>
<td>71 ± 9</td>
<td>64 ± 11*</td>
</tr>
<tr>
<td>60</td>
<td>63 ± 3</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>120</td>
<td>65 ± 7</td>
<td>79 ± 16</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>80 ± 6</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>15</td>
<td>339 ± 21</td>
<td>393 ± 25</td>
</tr>
<tr>
<td>0</td>
<td>429 ± 13</td>
<td>390 ± 20**</td>
</tr>
<tr>
<td>1</td>
<td>415 ± 12</td>
<td>398 ± 18*</td>
</tr>
<tr>
<td>15</td>
<td>460 ± 15</td>
<td>409 ± 18*</td>
</tr>
<tr>
<td>30</td>
<td>438 ± 16</td>
<td>379 ± 19**</td>
</tr>
<tr>
<td>60</td>
<td>426 ± 18</td>
<td>376 ± 23**</td>
</tr>
<tr>
<td>120</td>
<td>448 ± 8</td>
<td>391 ± 32**</td>
</tr>
<tr>
<td>Min</td>
<td>377 ± 20</td>
<td>379 ± 20</td>
</tr>
</tbody>
</table>

\* \( P < 0.05 \) compared with same time point in corresponding vehicle group.

\*\* \( P < 0.01 \) compared with same time point in corresponding vehicle group.

\* \( P < 0.05 \) compared with corresponding group of rats given 60 mg kg⁻¹ NCX4016.

\*\* \( P < 0.01 \) compared with nondiabetic controls.
controls. During reperfusion, there was no difference between any group and controls, although low-dose NCX4016-treated rats had a significantly higher MABP compared with those receiving high-dose NCX4016. Aspirin significantly reduced heart rate throughout the protocol in normal rats compared with controls (Table 1). None of the other drug treatments had any significant effect on HR during stabilization, ischemia, or reperfusion.

In diabetic animals, aspirin significantly reduced MABP compared with diabetic controls during stabilization (Table 1). During ischemia, animals receiving NCX4016 at either dose or aspirin all demonstrated a significantly reduced MABP compared with 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 compared with rats pretreated with high-dose NCX4016. As in normal rats, aspirin significantly reduced HR throughout the ischemia and reperfusion protocol compared with all groups. High-dose NCX4016 significantly reduced HR during ischemia, whereas low-dose NCX4016 only reduced HR during reperfusion (Table 1).

There was no significant difference in the total number of arrhythmias that occurred during ischemia between normal and diabetic rats (Fig. 1). There was also no difference between normal and diabetic animals in the time course of arrhythmic activity, with the majority of arrhythmias occurring between 6 and 15 min of ischemia in both normal and diabetic rats or in the incidence of severe arrhythmias (VT and ventricular fibrillation; Fig. 2). Neither aspirin nor NCX4016, at either dose, had any significant effects on the development of arrhythmias in normal rats. However, although 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 ($P < 0.05$) (Fig. 1). None of the drug treatments significantly modified the incidences of VT or ventricular fibrillation in normal rats, although NCX4016 reduced the incidence of VT ($P < 0.05$) in diabetic rats (Fig. 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 AAR (normal, 57 ± 3%; diabetic, 59 ± 4%) or infarcted area between normal (41 ± 4%) and diabetic (41 ± 6%) rats. AAR was not different in any of the drug treatment groups in the normal or diabetic rats (Fig. 3). Aspirin and the low dose of NCX4016 significantly reduced infarct size compared with 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, although neither aspirin nor the low dose of NCX4016 had any effect, the high dose of NCX4016 significantly reduced infarct size compared with controls.

**Biochemical Analysis.** There was no difference in circulating TxB2 concentrations between normal and diabetic control rats (3.6 ± 2.4 and 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 TxB2 production in blood from both normal (26.7 ± 3.4 ng ml$^{-1}$) and diabetic (20.7 ± 4 ng ml$^{-1}$) rats (Fig. 4). None of the drug treatments had any effect on circulating TxB2 levels in normal rats compared with controls. However, aspirin significantly reduced TxB2 production in response to ex vivo platelet activation with A23187 compared with controls, whereas NCX4016 had no effect on TxB2 production at either dose (Fig. 4A). In the diabetic rats, none of the drug treatments had any effect on circulating TxB2 levels compared with controls. Similar to the findings in blood from normal rats, aspirin significantly reduced TxB2 production in response to ex vivo activation with A23187 compared with controls.
whereas NCX4016 had no significant effect on TxB₂ production at either dose (Fig. 4B).

In normal rats, the plasma concentration of the aspirin breakdown product, salicylate, achieved in rats pretreated 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 pretreated 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 compared with 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 with an equimolar dose of aspirin (18.8 ± 3.9 versus 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 NOₓ (NO₂ + NO₃) concentrations between normal and diabetic control rats (Table 2). Five days of pretreatment with aspirin or the lower dose of NCX4016 had no significant effect in normal but the higher dose of NCX4016 significantly increased NOₓ concentration. However, in the diabetic rats, there was no difference in NOₓ 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 because it was abolished by thyroid replacement therapy or by...
insulin in a dose that normalized 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 hyperglycemia, reduced weight gain, polyuria, glycosuria, polyphagia, and polydipsia, while seeing only small decreases in plasma free thyroid hormone concentrations compared with those reported in untreated diabetic rats (Zhang et al., 2002). The high plasma glucose levels may reflect the time (24 h) between the last dose of insulin and measurement. In pilot studies when insulin was given 1 h before anesthesia, 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. Thus, it seems 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 after ischemia and reperfusion. Moreover, STZ diabetes had no effect on platelet activity, as evidenced by the absence of any difference between nondiabetic and diabetic rats in the generation of TxB₂ in response to A23187. Several studies have shown that platelets from diabetic animals are bigger (Judge et al., 1995) and produce more thromboxane B₂ 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 ischemia-induced arrhythmias in normal rats, whereas the higher dose of NCX4016, but not aspirin, reduced the number of ventricular ectopic beats after ischemia 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 ischemia-reperfusion cardiac arrhythmias to be reduced by both NCX4016 and aspirin in normal rats. These observations raise questions concerning the mechanism whereby NCX4016 exerts its protective effect and the greater effectiveness of NCX4016 compared with aspirin in this model of diabetes. Because NCX4016 did not modify plasma glucose concentrations, its effectiveness in diabetic animals was not due to modification of the diabetic state.

NCX4016 is rapidly metabolized 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 (Corazzi 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 after administration of NCX4016 compared with that achieved after an equimolar dose of aspirin. Second, aspirin, but not NCX4016, reduced thromboxane B₂ generation from blood samples in response to the calcium ionophore A23187. Some studies have similarly reported a lack of effect of NCX4016 on TxB₂ production by platelets in rats (Bak et al., 1998), whereas in other species, platelet TxB₂ production was inhibited by NCX4016 (Wainwright et al., 2002). Furthermore, NCX4016 does seem to be an effective antithrombotic agent 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 5 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 (S. G. Burke, I. Vojnovic, T. Warner, D. G. Watson, B. L. Furman, and C. L. Wainwright, unpublished data). Although 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 antiplatelet effect.

The cardioprotective action of NCX4016 may be due to the release of the nitric oxide moiety because 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 ischemia-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 nondiabetic rats after 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 to 6 h 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 because the concentrations were measured at only one time (3.75 h) after the last oral dose. Furthermore, NO released from NCX4016 can also be stored as nitrosylhemoglobin and S-nitrosothiols after 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 NOₓ is underestimating the amount of bioavailable NO. Comparison with an equivalent dose of a conventional nitric oxide donor, although 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 ischemia has been demonstrated previously in rat...
isolated perfused hearts subjected to global ischemia fol-
lowed by reperfusion (Wanna et al., 1995). Recent work has also shown that the effects of NCX4016 on neutrophil/endo-
thelium 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 ischemia and reperfusion with aspirin and NCX4016 may contribute to their beneficial effects by reducing oxygen de-
mand; aspirin reduced heart rate throughout ischemia and reperfusion in both diabetic and control rats, whereas NCX4016 reduced heart rate only during reperfusion with the high dose and only during ischemia for the low dose. An earlier study in conscious, chronically infected rats showed aspirin to reduce postinfarction heart rate, while maintain-
ing cardiac output (Schoemaker et al., 1998). Because 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 ischemia, 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 seemed 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 dependence upon endoge-
nous cardioprotective prostanooids generated during ex ischemia (Wainwright and 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 prostanooid protective mechanism.

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. NCX4016 may protect the diabetic heart through a mecha-
nism 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 dia-
betic patients may require modified dosages compared with nondiabetic patients.

Acknowledgments

We thank Graham Beastall (Institute of Biochemistry, Glasgow Royal Infirmary) for the assays of thyroid hormones and J. Brown and S. McDonald (Biological Procedures Unit, University of Strath-
clyde) for technical assistance.

References

Del Solpado P, Sorrentino R, and Pinto A (1999) NO-aspirins: a class of new anti-
Paulson DJ (1997) The diabetic heart is more sensitive to ischemic injury. Cardio-
vasc Res 34:104–112.
derivatives of aspirin, NCX 4016, reduce infarct size and protect against diabetes-
ible inhibition of cyclooxygenase-1 (COX-1) by nitroaspirin (NCX 4016). J Phos-
pharmacol 357:365–373.

Address correspondence to: Dr. Brian Furman, Strathclyde University, Strathclyde Institute of Biomedical Sciences, John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR, Scotland. E-mail: b.l.furman@strath.ac.uk