Abstract  Ticlopidine, a thienopyridine that prevents the progression of diabetic retinopathy in humans, was recently shown to increase nitric oxide (NO) production in human neutrophils. The thienopyridine clopidogrel has been found to be clinically useful in the secondary prevention of thrombotic events. The aim of the present study was to evaluate the effect of clopidogrel on ischemic retinopathy in streptozotocin-diabetic rats and its influence on prostanoids and NO production. We compared nondiabetic rats and rats after 3 months of diabetes that were given three doses (1, 10 or 20 mg/kg per day p.o.) of ticlopidine or clopidogrel from the first day of diabetes.

The variables recorded after 3 months of diabetes were platelet aggregation, thromboxane B₂ (TxB₂) production, 6-keto-prostaglandin F₁α (stable metabolite of prostacyclin), aortic NO, plasma nitrites/nitrates, and the percentage of the retinal surface occupied by horseradish peroxidase (HRP)-permeable vessels. In diabetic rats, platelet aggregation and thromboxane concentration were increased, and prostacyclin, NO and area occupied by HRP-permeable vessels were decreased.

Ticlopidine and clopidogrel reduced the maximum extent of platelet aggregation in a dose-dependent manner: maximal inhibition with respect to untreated diabetic rats was 48.6% with ticlopidine and 66.6% with clopidogrel. Ticlopidine reduced thromboxane B₂ only at a dose of 20 mg/kg per day p.o. (47.4% inhibition) and clopidogrel at doses of 10 mg/kg per day (51% inhibition) or 20 mg/kg per day (51.7% inhibition). Aortic prostacyclin production did not change after treatment with either thienopyridine. Treatment with ticlopidine reduced the inhibition of NO production in untreated rats (89.6% inhibition) to 0.9%, and clopidogrel reduced inhibition to 30%. Treatment with ticlopidine or clopidogrel reduced the retinal nonperfused area from 86.8% inhibition in untreated rats to 45.6% and 25.3%, respectively.

In conclusion, the early administration of thienopyridines in streptozotocin-diabetic rats partly prevented the appearance of diabetic retinal ischemia.

Keywords  Ticlopidine · Clopidogrel · Diabetic retinopathy · Nitric oxide · Prostacyclin

Introduction

Two of the mechanisms involved in the genesis and progression of microangiopathic complications in patients with diabetes mellitus and in experimental diabetic animals are increased platelet activation (Boeri et al. 2001; Dallinger et al. 1987; De La Cruz et al. 1997; Ishii et al. 1992) and increased thromboxane synthesis (De La Cruz et al. 1997; Hendra and Betteridge 1989; Moreno et al. 1995). A decrease in platelet sensitivity to endogenous platelet substances such as prostacyclin or nitric oxide (NO), and a decrease in the synthesis of these mediators, have also been described (De La Cruz et al. 2002b, 2002c; Hendra and Betteridge 1989). In diabetic retinopathy, these alterations are closely related with endothelial dysfunction, one of the earliest alterations to appear (Chakrabarti et al. 2000).

Some antiplatelet drugs such as aspirin (De La Cruz et al. 1990, 1997, 2002b), dipyridamole, m odpamil (De La Cruz et al. 1996), ditazol (Moreno et al. 1995), camognol and other antagonists of thromboxane synthase (De La Cruz et al. 1998, 2000b) have shown a prophylactic effect on the development and progression of these retinal vascular lesions in streptozotocin-diabetic rats. From these studies we concluded that these drugs exerted two effects that correlated with the prevention of ischemic di-
abetic retinopathy: inhibition of platelet thromboxane, and increased vascular production of prostacyclin and NO.

Ticlopidine is a thienopyridine that prevents the evolution of diabetic retinopathy in humans (TIMAD Study Group 1990), and it has recently been shown to increase NO production in human neutrophils (De La Cruz et al. 2002a). Another thienopyridine, clopidogrel, is used in the secondary prevention of thrombotic events (CAPRIE Steering Committee 1996). Ticlopidine and clopidogrel act as antagonists of platelet ADP receptors (Foster et al. 2001).

The aim of the present study was to evaluate the possible effect of clopidogrel on ischemic retinopathy in streptozotocin-diabetic rats, and its influence on prostanooids and NO production.

Materials and methods

Materials

All reagents were from Sigma Chemical (St. Louis, Mo., USA), unless otherwise noted. Isophane (NPH) insulin was obtained from Novo Nordisk ( Bagsvaerd, Denmark), and collagen was obtained from Menarini (Barcelona, Spain). Ticlopidine and clopidogrel were obtained from Sanofi-Synthelabo (Barcelona, Spain).

Experimental groups

We used 80 male Wistar rats with a mean body weight of 200 g at the start of the experiment. The study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals, and the research was approved by the University of Málaga Animal Use Committee.

The rats were distributed randomly into eight groups of ten animals each: (1) a control group of nondiabetic animals studied for 3 months, (2) an untreated group of animals with diabetes followed for 3 months, (3) animals with diabetes treated with 1 mg ticlopidine/kg per day p.o. for 3 months, (4) rats with diabetes treated with 10 mg ticlopidine/kg per day p.o. for 3 months, (5) animals with diabetes treated with 20 mg ticlopidine/kg per day p.o. for 3 months, (6) animals with diabetes treated with 1 mg clopidogrel/kg per day p.o. for 3 months, (7) rats with diabetes treated with 10 mg clopidogrel/kg per day p.o. for 3 months, (8) animals with diabetes treated with 20 mg clopidogrel/kg per day p.o. for 3 months.

Induction of diabetes

Experimental diabetes was induced with a single intravenous injection of 50 mg/kg streptozotocin. Blood glucose concentration was measured by placing a Glucocard Memory II glucometer (Menarini, Barcelona, Spain) in contact with blood from a small incision in the tail. Animals were considered to have diabetes if blood glucose was >200 mg/dl for 2 consecutive days. Rats in the nondiabetic control groups received a single intravenous injection of isotonic saline solution, and blood glucose was measured in the same way as in animals that were made diabetic.

Observation and treatment

During the observation period, diabetic animals were treated with 4 IU/day s.c. of NPH insulin to reduce mortality due to the high levels of blood glucose. Control animals received the same volume of isotonic saline solution s.c.

Drugs were given starting on the first day of diabetes as a single oral daily dose via a flexible catheter. Nondiabetic control animals received an equivalent volume of isotonic saline solution.

Sample processing

At the end of the third month all animals from each group were anesthetized with pentobarbital sodium (40 mg/kg i.p.). A medial laparotomy was made to withdraw 2 ml of blood from the vena cava; 3% sodium citrate at a proportion of 1:9 was used as the anticoagulant. Then a segment of the abdominal aorta 0.5 cm anterior to the bifurcation of the femoral arteries was clamped.

Sigma Type II horseradish peroxidase (HRP; 1 ml, 180 mg/kg) was injected via the carotid artery. Five minutes later both eyeballs were removed and placed in a solution of 1.2% glutaraldehyde and 1% paraformaldehyde in 0.2 M phosphate-buffered saline (pH 7.2) for 45 min. The lens and vitreous humor were removed, and the retina was separated from the sclera with a narrow surgical spatula and immersed in fixative for 48 h.

Analytical techniques

All techniques were run in a single-blind manner, i.e., the persons who did the assays were unaware of the origin and nature of the samples.

Platelet aggregometry. Platelet aggregation capacity in whole blood was tested at 37°C with the electrical impedance method (Cardinal and Flower 1980). Collagen (10 µg/ml) was used as the inducing agent, and maximum aggregation intensity was determined as the maximum resistance between the two poles of the electrode obtained 10 min after collagen was added.

Platelet thromboxane B2. After aggregation was complete the blood sample was centrifuged at 10,000 g for 5 min, and the supernatant was frozen at –80°C until thromboxone B2 production was quantified with an enzyme immunoassay (Oxford Biomedical Research, Oxford, Mich., USA).

Vascular 6-keto-prostaglandin F1α. The aortic segment was cut into two parts and incubated at 37°C in buffer containing (mM): 100 NaCl, 4 KCl, 25 NaHCO3, 2.1 Na2SO4, 20 sodium citrate, 2.7 glucose and 50 Tris (pH 8.3). One segment was placed in 500 µl of fresh buffer, and 10 µl calcium ionophore A23187 (final concentration 1 µM) was added. Five minutes later the sample was dried and weighed, and the supernatant was frozen at –80°C until the assay. The production of 6-keto-prostaglandin F1α (6-keto-PGF1α; stable metabolite of prostacyclin) was quantified with an enzyme immunoassay (Oxford Biomedical Research).

Vascular nitric oxide production. The other part of the aortic segment was incubated in fresh buffer, and 100 µM L-arginine was added. Nitric oxide production was quantified by an electrochemical method (Shibuki 1990), with an ISO-NOP 200 electrode for NO detection (World Precision Instruments, Stevenage, UK). Production was induced with 1 µM calcium ionophore A23187 to stimulate constitutive NO-synthase.

Plasma nitrite/nitrate levels. As an indirect indicator of overall NO production in each animal, we determined plasma nitrite/nitrate levels. One milliliter of blood (with anticoagulant) was centrifuged at 10,000 g for 10 min, and the supernatant was filtered through Ultrapure MC microcentrifuge filters (Gif-sur-Yvette, France) to eliminate hemoglobin released by cell lysis. The nitrite/nitrate level was measured with a commercial kit (Cayman Chemical, Ann Arbor, Mich., USA), based on the Griess reaction, after the nitrates were converted to nitrates with nitrate reductase. Levels of nitrite/nitrate were determined spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite.