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ScienceDirect
Vascular Pharmacology 47 (2007) 83 – 89
Vascular Pharmacology
www.elsevier.com/locate/vph

Gender differences in the effect of aspirin on retinal ischemia, prostanoid synthesis and nitric oxide production in experimental type 1-like diabetes

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Received 1 July 2006; accepted 1 November 2006

Abstract

Background: The protective effect of acetylsalicylic acid (aspirin) against cardiovascular events is known to be weaker in women than in men. The present study was designed to test whether this effect of aspirin differed between sexes in an experimental model of diabetes with retinal ischemia.
Methods: We compared nondiabetic rats and rats after 1, 2 and 3 months of diabetes that were given 2 mg/kg/day p.o. of aspirin from the first day of diabetes. The variables recorded were platelet aggregation, production of thromboxane B_2 (TxB_2), 6-keto-prostaglandin $F_{1\alpha}$ and aortic nitric oxide, and the percentage of the retinal surface occupied by horseradish peroxidase (HRP)-permeable vessels.
Results: In female rats made diabetic, TxB_2 synthesis was more markedly reduced, and the percentage of HRP-permeable retinal vessels was less markedly reduced, than in their male counterparts. The response to aspirin treatment was weaker in female than in male diabetic rats in terms of inhibition of TxB_2 synthesis, increased nitric oxide production, and prevention of the increase in the percentage of retinal surface covered by HRP-permeable vessels.
Conclusion: Aspirin was less effective in preventing retinal ischemia in experimental diabetes in female than in male rats.

Keywords: Aspirin; Diabetes; Diabetic retinopathy; Gender

1. Introduction

Cardiovascular ischemic disease, specifically ischemic heart disease and ischemic stroke, are more frequent in men than in premenopausal women, although percentage incidence and prevalence are similar in both genders when postmenopausal women are considered. However, the prognosis and clinical course of these cardiovascular events is worse in women.

One of the factors that obviates the differences between genders in the course of cardiovascular disease is diabetes mellitus (Hu et al., 2001; Kanaya et al., 2002; Natarajan et al., 2003). The tendency for differences between men and women to disappear when diabetes is present is paralleled by differences in the approach to treatment for cardiovascular disease for men and women (Wexler et al., 2005), both at the primary care level (Williams et al., 2003) and in hospital care (Rathore et al., 2001). In general, women receive less aggressive treatment (Corbelli et al., 2003; Daly et al., 2006).

Acetylsalicylic acid (ASA) has been found to be have differently in women in terms of its antithrombotic effect, with the result that it offers less protection against cardiovascular events (Levin, 2005), a difference that may be related to the higher rate of nonresponse to ASA in women (Alberts et al.,

Abbreviations: ASA, acetylsalicylic acid; HRP, horseradish peroxidase; NO, nitric oxide; TxB_2, thromboxane B_2.

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doi:10.1016/j.vph.2006.11.007

2004; Gum et al., 2001) or to differences between genders in metabolism of the drug in the liver (Ho et al., 1985; Vargas Loza et al., 1997). It has also been postulated that in women, platelets behave differently in terms of their aggregating response in comparison to men both under basal conditions and in response to ASA (De La Cruz et al., 1986; Leng et al., 2004).

Most studies to date have investigated the use of ASA to treat macrovascular thrombotic complications, but some research has also supported its use to prevent experimental microangiopathies such as diabetic retinopathy (De La Cruz et al., 1997, 2004a,b; Sun et al., 2005). The aim of the present study was to determine whether the effect of ASA in an experimental model of retinal ischemia caused by diabetes mellitus differed in any way between male and female animals. We compared indicators of the main actions of ASA (platelet aggregation, prostanoid synthesis, and vascular nitric oxide [NO] production), and quantified the effects of the drug on retinal ischemia.

2. Methods

2.1. Material

All reagents including acetylsalicylic acid were from Sigma Chemical Corp. (St. Louis, MO, USA), unless otherwise noted. Isophane (NPH) insulin was obtained from Novo Nordisk (Bagsvaerd, Denmark), and collagen was obtained from Menarini, S.A. (Barcelona, Spain).

2.2. Experimental groups

We used 180 Wistar rats (90 male and 90 female) 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 Malaga Animal Use Committee.

The rats were distributed randomly into 9 groups of 20 animals each (10 male and 10 female): (1) a control group of nondiabetic animals studied for 1 month, (2) a control group o f nondiabetic animals studied for 2 months, (3) a control group of nondiabetic animals studied for 3 months, (4) an untreated group of animals with diabetes followed for 1 month, (5) an untreated group of animals with diabetes followed for 2 months, (6) an untreated group of animals with diabetes followed for 3 months, (7) animals with diabetes treated with 2 mg ASA/kg/day p.o. for 1 month, (8) animals with diabetes treated with 2 mg ASA/kg/day p.o. for 2 month, (9) animals with diabetes treated with 2 mg ASA/kg/day p.o. for 3 months.

2.3. 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 glucosimeter (Menarini, SA, 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.

2.4. 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.

2.5. Sample processing

At the end of each period 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.

2.6. 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.

2.6.1. 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.

2.6.2. Platelet thromboxane B_2

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 thromboxane B_2 (TxB_2) production was quantified with an enzyme immunoassay (Oxford Biomedical Research Inc., Oxford, MI, USA).

2.6.3. Vascular 6-keto-prostaglandin $F_{1\alpha}$

The aortic segment was cut into two parts and incubated at 37 °C in buffer containing (mM) 100 NaCl, 4 KCl, 25 $NaHCO_3$, 2.1 Na_2SO_4, 20 sodium citrate, 2.7 glucose and 50 Tris (pH 8.3). One segment was placed in 500 μL fresh buffer, and 10 μL

Table 1
Body weight, blood glucose and hematocrit after 1, 2 and 3 months ($N=10$ rats per group) in nondiabetic rats (NDR), diabetic rats without treatment (DR) and DR treated with 2 mg/kg/day p.o. aspirin (DR+ASA)

	Body weight (g)			Blood glucose (mg/dL)			Hematocrit (%)		
	1 month	2 months	3 months	1 month	2 months	3 months	1 month	2 months	3 months
NDR									
Male	215±19.5	325±28.8	435±25.1	82.2±2.1	81.3±3.2	80.0±5.4	45.1±0.4	45.3±0.6	45.2±0.6
Female	210±15.6	335±27.5	406±21.2	84.6±5.1	84.1±6.2	83.4±4.1	44.8±0.8	44.5±1.2	45.1±0.8
DR									
Male	278±16.1	292±18.1	298±19*	479±15*	432±26*	455±19*	50.0±1.6*	53.8±2.2*	48.3±2.2
Female	265±12.0	283±25.7	287±21*	487±19*	429±17*	445±16*	48.5±2.0*	49.3±3.1*	46.5±40
DR+ASA									
Male	331±28.4	344±22.8	332±14.3	469±18*	468±16*	485±10*	49.4±1.7*	53.1±2.0*	50.1±1.4*
Female	320±17.2	348±19.1	345±19.8	484±14*	450±23*	471±22*	48.5±1.1*	49.7±2.0*	47.2±1.1

*$P<0.05$ with respect to NDR.

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 $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (stable metabolite of prostacyclin) was quantified with an enzyme immunoassay (Oxford Biomedical Research, Inc.).

2.6.4. 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 Ltd., Stevenage, Herts., UK). Production was induced with 1 μM calcium ionophore A23187 to stimulate constitutive NO-synthase.

2.6.5. Retinal vasculature

The fixed retinas were incubated with a solution of tetramethylbenzidine and sodium nitroferricyanide as the chromogenic substrate, then dehydrated in an alcohol gradient, incubated in xylene, and mounted in sections for microphotography.

Retinal vessels permeable to HRP were photographed at ×40, and microscopic images were processed in an IBAS Kontron 2000 image analyzer (Kontron Bidanalyse, Munich, Germany). The percentage of the retinal surface occupied by HRP-permeable vessels was calculated by the system as a standard parameter. Horseradish peroxidase is a high-molecular-weight substance that mainly stains red blood cells; thus in occluded vessels HRP does not stain the vasculature.

2.7. Statistical analysis

All values in the text and figures are the mean ± standard error of the mean (SEM) of the data for all animals in each group. The data were analyzed with the Statistical Package for Social Sciences (SPSS Co., Chicago, IL, USA). Groups were compared with analysis of variance followed by the test of minimum significant differences when the difference between groups was significant. A P value of <0.05 was taken as the minimum level of significance.

3. Results

Body weight in diabetic rats was significantly lower than in nondiabetic animals after 3 months (Table 1). We found no significant differences between male and female rats. Blood cell

Table 2
Maximum intensity of collagen-induced platelet aggregation in whole blood (Imax) and thromboxane B_2 (TxB_2) production induced with 1 μM calcium ionophore A 23187 ($N=10$ rats per group) in nondiabetic rats (NDR), diabetic rats without treatment (DR) and DR treated with 2 mg/kg/day p.o. aspirin (DR+ASA)

	Imax (Ω)			TxB_2 (nmol/10^9 platelets)		
	1 month	2 months	3 months	1 month	2 months	3 months
NDR						
Male	5.7±0.6	5.8± ±0.6	6.2±0.5	0.96±0.09	0.98±0 .09	0.94±0.09
Female	5.5±0.4	5.5±0.6	6.0±0.8	0.67±0.07‡	0.69±0 .08‡	0.61±0.08‡
DR						
Male	6.9±0.5 *	9.3±0.9**	15.9±1.8**	2.54±0.16**	7.43±0 .95**	9.08±0.79**
Female	6.6±0.7 *	9.1±0.7**	16.0±1.8**	1.43±0.16‡**	5.57±0 .48‡**	9.80±0.81**
DR+acetylsalicylic acid						
Male	0.5±0.02†	0.2±0.02†	0.3±0.02†	0.25±0.04†	0.31±0 .02†	0.30±0.04†
Female	0.5±0.03†	0.3±0.02†	0.3±0.03†	0.54±0.07‡†	0.53±0.06‡†	0.41±0.03‡†

*$P<0.05$, **$P<0.001$ with respect to NDR. †$P<0.0001$ with respect to DR. ‡$P<0.05$ with respect to value in male rats.

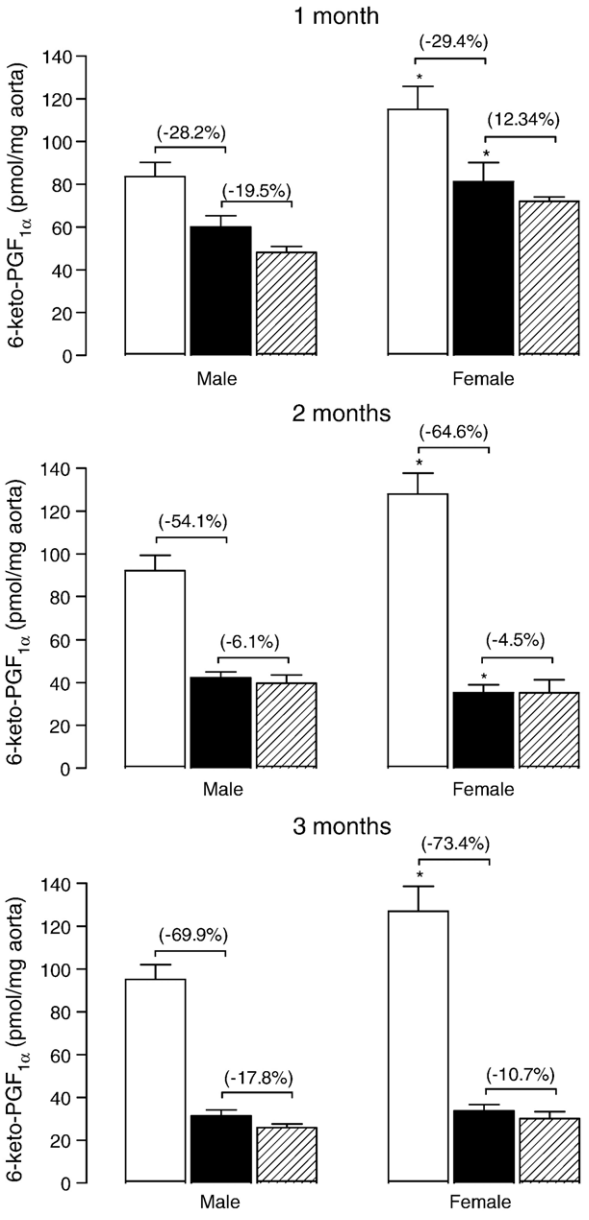

Fig. 1. Vascular 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) production in ($N=10$ rats per group) in nondiabetic rats (white bars), diabetic rats without treatment (black bars) and diabetic rats treated with 2 mg/kg/day p.o. aspirin (hatched bars). *$P<0.05$ with respect to male rats.

counts were not significantly changed in animals with diabetes (data not shown), the only significant difference between groups being the higher hematocrit compared to normoglycemic animals in diabetic rats with and without treatment (Table 1). No differences were found between male and female rats. As expected, glucemia values were significantly higher in animals made diabetic than in nondiabetic controls, but there were no differences in glucemia values in diabetic rats between sexes or between different study periods (Table 1).

Table 2 shows the data for platelet-related parameters maximum intensity of collagen-induced platelet aggregation and TxB_2 production. Maximum platelet aggregation was significantly higher in diabetic animals than in controls after 2 and 3 months, with no significant differences between sexes. Treatment with ASA reduced these values significantly, with no differences between males and females at any time period.

Thromboxane B_2 production was significantly lower in female than in male normoglycemic rats, and was higher in diabetic than in nondiabetic animals as early as after the first month of follow-up. In the three groups, TxB_2 production was significantly lower in females. Treatment with ASA significantly reduced TxB_2 production, although the decrease was significantly larger in male rats.

Vascular prostacyclin production was significantly greater in female than in male normoglycemic rats. In animals made diabetic, vascular 6-keto-$PGF_{1\alpha}$ production was significantly

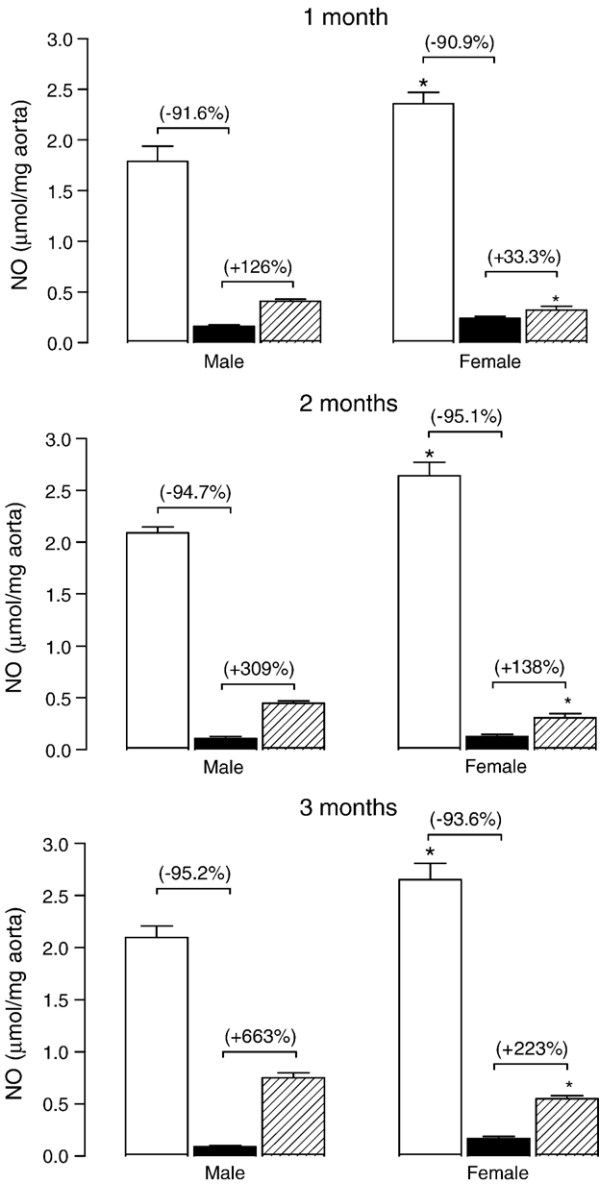

Fig. 2. Vascular nitric oxide (NO) production in ($N=10$ rats per group) in nondiabetic rats (white bars), diabetic rats without treatment (black bars) and diabetic rats treated with 2 mg/kg/day p.o. aspirin (hatched bars). *$P<0.05$ with respect to male rats.

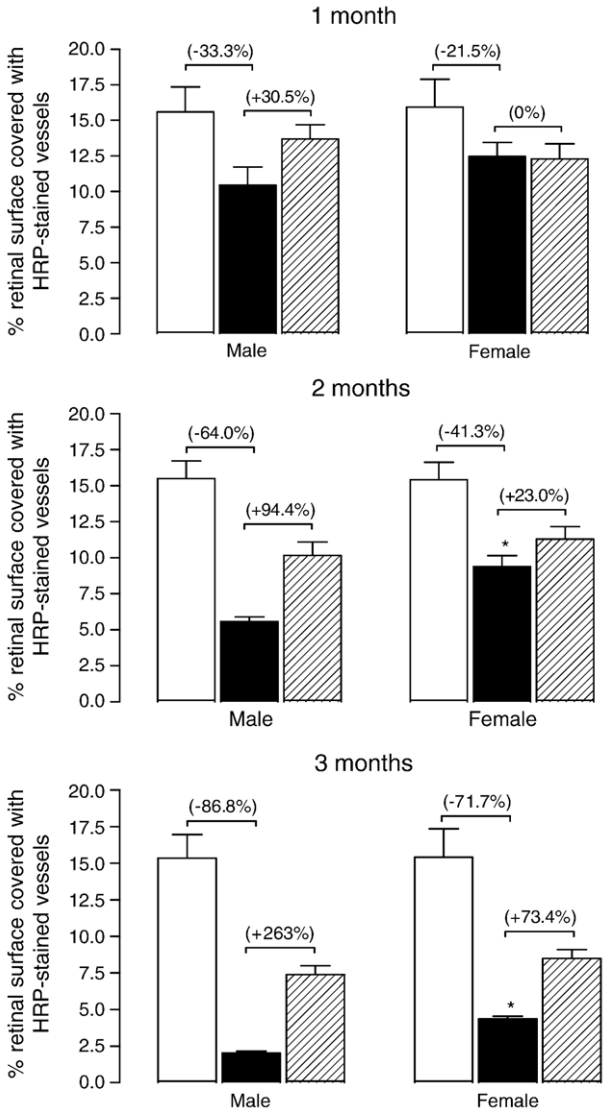

Fig. 3. Percentages of retinal surface covered with horseradish peroxidase (HRP)-stained vessels in (N=10 rats per group) in nondiabetic rats (white bars), diabetic rats without treatment (black bars) and diabetic rats treated with 2 mg/kg/day p.o. aspirin (hatched bars). *P<0.05 with respect to male rats.

lower than in nondiabetic animals, and the extent of the reduction was similar in both sexes. Aspirin treatment slightly decreased prostacyclin synthesis to a similar extent in both sexes after 1, 2 and 3 months (Fig. 1).

Vascular NO production was significantly greater in aortic tissue from female than male normoglycemic rats. In animals made diabetic, vascular NO production was significantly reduced (Fig. 2) to a similar degree in both sexes. However, the increase in NO production with ASA treatment was significantly greater in male rats (Fig. 2).

In animals made diabetic, the percentage of retina occupied by HRP-permeable vessels decreased significantly in a time-dependent manner (Fig. 3). The decrease was greater in male than in female rats. Treatment with ASA significantly reduced the loss of vascular filling caused by diabetes, but this effect was significantly weaker in female rats (Fig. 3).

4. Discussion

The present study yields three main findings. First, we found differences between male and female normoglycemic rats: platelet thromboxane synthesis was lower in females, and vascular prostacyclin and NO production were higher. Second, in animals made diabetic, thromboxane synthesis was more clearly affected and the percentage of retina occupied by HRP-permeable vessels was less markedly altered in females. Third, in animals treated with ASA, the response in females was weaker than in males, particularly when measured as the inhibition of TxB_2 synthesis, the increase in NO production and the prevention of alterations in the percentage of retina occupied by HRP-permeable vessels.

In normoglycemic rats the differences we found between sexes are consistent with earlier findings that biochemical pathways of antithrombotic action and vasodilation, such as prostacyclin and NO, show a greater activity in females. Constitutive nitric oxide synthase activity (cNOS) has been reported to be higher in female animals (Weiner et al., 1994), a finding consistent with our results, since cNOS activity is calcium-dependent, as is the experimental stimulus we used to induce vascular NO production. In addition, the NO-cGMP and prostacyclin-cAMP pathways have been found to be more active in female animals, and to lead to greater sensitivity to vasodilating stimuli in the arteries of female animals than in males (Lu et al., 2005; Mayhan et al., 2002; Thompson and Khalil, 2003). These differences have been explained as an effect of estrogens and progestogens on the synthesis of these mediators (Egan et al., 2004; Lu et al., 2005; Mayhan et al., 2002; Orshal and Khalil, 2004; Thompson and Khalil, 2003; Weiner et al., 1994).

The changes we found in male and female animals made diabetic in comparison to normoglycemic animals are consistent with the findings of earlier studies with the same experimental model.

Enhanced platelet aggregation in diabetes has been widely reported in both animal models and humans. As earlier studies have shown (Glassman, 1993; Schror, 1997), this increase in platelet aggregation capacity is accompanied by an evident imbalance in prostanoid synthesis, i.e., an increase in platelet thromboxane A_2 production and a decrease in vascular prostacyclin synthesis.

With respect to NO production, sustained high blood glucose levels in diabetes mellitus are assumed to stimulate oxidative stress mechanisms, which may be one of the causes of the inhibition in endothelial prostacyclin synthetase and the reaction of free radicals with NO to produce peroxy nitrites (Giugliano et al., 1996; Tesfamariam and Cohen, 1992). Both effects are manifested as the endothelial dysfunction reported in diabetes. Both prostacyclin and NO are vasodilators and platelet antiaggregants, and a deficit in these substances has been linked to early vascular alterations in diabetic retinopathy (Chakrabarti et al., 2000). In our experimental model, vascular NO synthase activity (measured indirectly as NO production after stimulation with a calcium ionophore) was clearly reduced in diabetic animals, as was the plasma nitrite/nitrate level, used here as an indirect indicator of global NO production in these animals.

The alterations summarized above may contribute to the reduction we found in retinal surface area occupied by HRP-

permeable vessels. However, these are not the only mechanisms that can condition retinal ischemia in diabetes. It is now accepted that the appearance of ischemia may reflect the sum of these and other factors such as increased endothelin synthesis, anomalies in protein kinase C, and increased activity of the polyol pathway (De Vriese et al., 2000; Chakrabarti et al., 2000). However, the synthesis of both prostacyclin and NO is altered as a result of the endothelial dysfunction in diabetes.

There were no appreciable differences between male and female animals in the changes in these parameters as a result of diabetes. In females we noted that the increase in platelet TxB_2 synthesis was smaller than in males at 1 and 2 months of follow-up, and ischemic changes tended to be smaller. These findings are consistent with published studies (Brown et al., 2001; Mayhan et al., 2002) that reported that the differences between male and female normoglycemic animals (lower vascular and cardiac contractility in female rats) disappeared in diabetic animals, since the alterations caused by diabetes were similar in both sexes. In the present study we found lower platelet TxB_2 synthesis and higher vascular prostacyclin production in female normoglycemic rats; these differences, however, tended to disappear with time (over a period of 3 months).

With regard to possible sex differences in the effects of ASA in our experimental model, our results showed that the changes in response to the drug were smaller in female than in male rats. Specifically, the inhibition of platelet cyclooxygenase, the increase in NO production, and the prevention of the diabetes-induced effect on the retinal vasculature were all weaker in female animals.

The smaller effect of ASA on platelet function in females has been reported in several species. In rats the ability of ASA to inhibit the mobilization of platelet calcium was weaker (Morikawa et al., 1986); in rabbits the ASA-induced reduction in fibrin formation in vivo was smaller (Kelton et al., 1978); and in humans ASA inhibited platelet-subendothelium interactions in a perfusion system to a lesser extent in women than in men (Escolar et al., 1986), and the 50% inhibitory concentration (IC_{50}) of ASA was higher (lower potency of its platelet antiaggregant effect) in blood from women (De La Cruz et al., 1986).

A number of pharmacokinetic or pharmacodynamic factors may contribute to the weaker effects of ASA in women. Studies of drug kinetics have shown that the bioavailability and metabolism of ASA differ between sexes, with bioavailability being greater in women because of the higher rate of conjugation with glucuronic acid and glycine in men (Miners et al., 1986). Other studies found that in normoglycemic rats, enzymatic hydrolysis of ASA in the liver is greater in males than in females, whereas in the digestive tract mucosa and in plasma, hydrolysis rates are higher in females, although it should be noted that plasma hydrolysis was only 0.7% to 4% of the rate of hydrolysis in the liver in these animals (Vargas Loza et al., 1997). Similar proportions were found for aspirin–esterase activity in rats, in which enzyme activity in serum was 0.02%–0.05% of the activity in the liver (Benedito, 1998). In both studies and in light of the importance of the liver in ASA metabolism, it was thought that salicylic acid formed more rapidly in males than in females. However, this difference would not account for the lower effect of ASA in female rats. Even in the light of the influence of metabolism in the liver in these sex differences, pharmacokinetic mechanisms alone cannot account for the difference in the effects of the drug on platelet function in experiments done in vitro, where the liver plays no relevant role (De La Cruz et al., 1986).

Pharmacodynamic mechanisms could be invoked to explain the differences in the effects of ASA via different mechanisms in female and male rats. However, studies published to date give no indication of any differences between sexes in the mechanisms of action of ASA with regard to the activity of different cyclooxygenases or nitric oxide synthases. This lack of pharmacodynamic evidence suggests that the sex differences in experimental diabetic retinopathy may reflect other effects of ASA. Some of the effects that may be involved are its ability to stimulate the formation of antiinflammatory factors such as 15-epi-lipoxin (Chiang et al., 2006) and inhibit transcriptional factor C/EPB-β phosphorylation (Sun et al., 2005), or a difference it its ability to forestall damage caused by tissue oxidative stress (De La Cruz et al., 2004a,b; Gonzalez-Correa et al., 2006; Guerrero et al., 2004; Podhaisky et al., 1997).

As a final consideration, it was recently found that resistance to ASA is greater in patients with insulin-dependent diabetes mellitus than in the nondiabetic population. (The experimental model we used here is similar to insulin-dependent diabetes.) Moreover, among people with diabetes, the frequency of resistance to ASA is greater in women than in men (Mehta et al., 2006). The mechanisms underlying these differences remain unknown, and further work is needed to shed light on the effect of ASA in preventing diabetic retinopathy in men and women.

Acknowledgments

We thank D. Antonio Pino Blanes for his valuable technical assistance, and K. Shashok for translating the manuscript into English.

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