Antioxidant and antiplatelet effects of the alpha-tocopherol–aspirin combination in type 1-like diabetic rats

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Abstract

We analyze the effect of the combination of acetylsalicylic acid (2 mg/kg/day p.o.) and alpha-tocopherol (25 mg/kg/day p.o.) in a type-1-like experimental model of diabetes mellitus on platelet factors, endothelial antithrombotic factors and tissue oxidative stress. In diabetic rats, the combination of drugs had a greater inhibitory effect on platelet aggregation than in untreated control animals with diabetes (88.87%). The combination of drugs had little effect on the inhibition of thromboxane production (−90.81%) in comparison to acetylsalicylic acid alone (−84.66%), potentiated prostacyclin production (+162%) in comparison to alpha-tocopherol alone (+30.55%), and potentiated nitric oxide production (+241%) in comparison to either drug alone (acetylsalicylic acid +125%, alpha-tocopherol +142%). The combination of the two drugs improved the thromboxane/prostacyclin balance (0.145±0.009) in comparison to untreated diabetic animals (4.221±0.264) and in untreated healthy animals (0.651±0.045). It did not potentiate the antioxidant effect of either drug alone, but did increase tissue concentrations of reduced glutathione, especially in vascular tissue (+90.09% in comparison to untreated animals). In conclusion, in the experimental model of diabetes tested here, the combination of acetylsalicylic acid and alpha-tocopherol led to beneficial changes that can help protect tissues from thrombotic and ischemic phenomena.

Keywords: Aspirin; Vitamin E; Diabetes; Platelets; Oxidative stress

Introduction

Diabetes mellitus is the most frequent endocrinological disease in the western world. Its importance lies not only in its high prevalence, but also in the multifactorial nature of its onset, evolution and especially its complications. Type 1 diabetes generally progresses toward chronic microvascular complications in the retina (diabetic retinopathy), kidney (diabetic nephropathy) and peripheral nerves (diabetic neuropathy), whereas type 2 diabetes is associated with a higher incidence of macroangiopathic complications, i.e., pronounced and earlier atheromatous disease than in the nondiabetic population. However, it has been recently demonstrated that the incidence of macrovascular disease in type 1 diabetes mellitus is nearly equal to that in type 2 diabetes (Laing et al., 2003).

Pharmacological prevention of the macrovascular complications of diabetes is based on the use of platelet antiaggregants, among which the most widely used in daily clinical practice is acetylsalicylic acid (ASA) (Antithrombotic Trialists’ Collaboration, 2002). In the face of limited pharmacological resources, one way to enhance the prevention of complications in vascular tissues is to associate drugs that act via different pathways that play a basic role in the development of vascular complications.

In diabetes mellitus oxidative stress is increased, a situation that favors atherothrombotic processes, thrombogenesis, and endothelial dysfunction (Aydin et al., 2001). Epidemiological studies have shown that dietary supplementation with alphatocopherol, a “standard” among antioxidant drugs, can reduce lipid peroxidation (Devaraj et al., 1997), prevent endothelial dysfunction (Keaney et al., 1996), and modulate both platelet adhesion and aggregation (Higashi and Kikuchi, 1974).

A potential alternative for therapy may thus consist of the combination of ASA and alpha-tocopherol. Recent studies have
shown this combination to potentiate the effect of ASA in platelet-rich plasma (Celestini et al., 2002) and whole blood (González-Correa et al., 2005), and to prevent transitory ischemic attacks (Steiner et al., 1995).

The main aim of this study was to analyze the effect of alpha-tocopherol on the antiplatelet action of ASA in a model of type-1-like experimental diabetes, and to evaluate the antioxidant effect of the combination of ASA and alpha-tocopherol on the tissues that are affected to the greatest extent by diabetes.

Materials and methods

Materials

All reagents were from Sigma Chemical Corp. (St. Louis, MO, USA) unless otherwise noted. Collagen was obtained from Menarini, S.A. (Barcelona, Spain). Thromboxane B₂ and 6-keto-PGF₁α ELISA kits were purchased from Oxford Biomedical Research Inc. (Oxford, MI, USA). The nitrite/nitrate ELISA kit was obtained from Cayman Chemical (Ann Arbor, MI, USA).

Study design

We used 50 male Wistar rats (CRIFFA, Charles River, Lyon, France) 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 5 groups of 15 animals each: (A) a control group of nondiabetic animals studied for 2 months, (B) an untreated group of animals with diabetes followed for 2 months, (C) animals with diabetes treated with 2 mg aspirin/kg/day p.o. for 2 months, (D) rats with diabetes treated with 25 mg alpha-tocopherol/kg/day p.o. for 2 months, and (E) animals with diabetes treated with 2 mg aspirin/kg/day p.o. plus 25 mg alpha-tocopherol/kg/day p.o. for 2 months. Most studies with alpha-tocopherol in rats use doses between 125 and 500 mg/kg in order to prevent oxidative stress damage; we have chosen 250 mg/kg/day p.o. according to these studies and previous experiments (data not shown) in our laboratory.

Induction of diabetes

Experimental diabetes was induced with a single intravenous injection of 50 mg/kg streptozotocin dissolved in citrated saline buffer diluted 1:50 with isotonic saline solution (final volume: 1 ml/kg). Blood glucose concentration was measured by placing a Glucocard Gmeter glucosimeter (Menarini S.A., 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 period of observation, diabetic animals were treated with 4 IU/day s.c. of NPH insulin to reduce mortality from 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 (4.5 ± 0.2 days from streptozotocin injection) as a single daily oral dose via a flexible catheter. Nondiabetic control animals received an equivalent volume of isotonic saline solution.

Sample processing

At the end of the second month all animals from all the groups were anesthetized with pentobarbital sodium (40 mg/kg i.p.). A medial laparotomy was made to withdraw blood from the vena cava; 3% sodium citrate at a proportion of 1:10 was used as the anticoagulant. The thoracic and abdominal aorta, liver, both kidneys and brain were removed and frozen at −80 °C until the experiments.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Values (mean±S.E.M.) for zoometric and blood parameters at the end of the 2-month study period in nondiabetic rats (NDR) and rats with diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDR</td>
<td>DR</td>
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<tr>
<td>Body weight (g)</td>
<td>340±14.29</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>13.77±0.44</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>2.13±0.05⁎</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>1.33±0.05</td>
</tr>
<tr>
<td>Glycemia (mg/dl)</td>
<td>117±3.72⁎</td>
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<tr>
<td>Red blood cells (× 10¹²/l)</td>
<td>6.79±0.69</td>
</tr>
<tr>
<td>Leukocytes (× 10⁹/l)</td>
<td>5.14±0.23</td>
</tr>
<tr>
<td>Platelets (× 10¹²/l)</td>
<td>678±93.7</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>13.57±0.13</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>34.43±0.73</td>
</tr>
<tr>
<td>Mean platelet volume (fl)</td>
<td>5.10±0.11</td>
</tr>
</tbody>
</table>

Shown are the data for untreated animals (DR) and for animals treated orally for 2 months with 2 mg/kg/day acetylsalicylic acid (ASA), 25 mg/kg/day alpha-tocopherol (AT), or both drugs together at the same doses. In each group n=15 rats.

* P<0.0001 in comparison to all other groups
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 aggregometry was performed in whole blood samples at 37 °C using the electrical impedance method (Chrono-Log aggregometer, Izasa S.A., Madrid, Spain) (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 completed, the blood sample was centrifuged at 10,000×g for 5 min, and the supernatant was frozen at −80 °C until thromboxane B₂ (stable thromboxane A₂ metabolite) production was quantified with a commercial immunoassay kit.

Vascular 6-keto-prostaglandin F₃α

An aortic ring was 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 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 F₃α (6-keto-PGF₁α, stable metabolite of prostacyclin) was quantified with a commercial immunoassay kit.

Plasma nitrite+nitrate levels

As an indirect indicator of overall nitric oxide (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
Ultrafree MC microcentrifuge filters to remove hemoglobin released by cell lysis. The nitrite/nitrate level was measured with a commercial kit based on the Griess reaction, after the nitrates were converted to nitrites with nitrate reductase. Levels of nitrite+ nitrate were determined spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite.

Lipid peroxidation

To quantify lipid peroxidation we measured thiobarbituric acid reactive substances (TBARS). Cell membrane-enriched fractions of the tissue samples were obtained as described previously (De La Cruz et al., 1992). Absorbance was determined spectrophotometrically at 532 nm (FluoStar, BMG Labtechnologies, Offenburg, Germany). The protein concentration was determined with the method of Bradford (1976). The final results are reported as nmol TBARS/mg protein; in red blood cells results are done as mmol TBARS/g hemoglobin.

Glutathione levels

Total glutathione was measured spectrofluorometrically according to the technique described by Hissin and Hill (1976). The tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid, then centrifuged at 13,000×g for 15 min at 4 °C to obtain the supernatant. Duplicate cuvettes were prepared for spectrophotometry with sodium phosphate buffer, the supernatant for each sample, and o-phthalaldehyde. To determine the proportions of oxidized and reduced glutathione we incubated the supernatant from each sample with 4-vinyl pyridine, then proceeded as for total glutathione. Results are reported as μmol GSH or GSSG per g tissue; in red blood cells results are done as μmol GSH or GSSG per g hemoglobin.

Enzyme activities related to glutathione

Glutathione peroxidase (GSHpx), glutathione reductase (GSSGrd) and glutathione transferase (GSHtf) were determined by spectrophotometric kinetics. Tissue samples were diluted in 0.1 M phosphate-buffered saline (pH 7.0) and 25% phosphoric acid. The mixture was homogenized and centrifuged at 13,000×g for 15 min at 4 °C. The supernatant was used to determine protein concentration after neutralization with 0.1 N NaOH, and to determine the following enzymatic activities.

Glutathione peroxidase

GSHpx activity was measured according to the method of Flohé and Gunzler (1984). Briefly, a volume equivalent to 25 μg protein was taken from each supernatant and 0.1 M phosphate-buffered saline was added to make up a volume of 880 μL, together with 34 mg/ml glutathione reductase, 3 mg/ml GSH, 1.25 mg/ml nicotinamide-adenine dinucleotide phosphate (NADPH) and 0.15 μL/ml terbutyl-hydroperoxide. The preparation was read at 340 nm and the decrease in absorbance was recorded every 30 s for 5 min.

Glutathione reductase

GSSGrd activity was determined according to the technique described by Flohé and Gunzler (1984). The amounts of sample and buffer were the same as in the GSHpx analyses. One hundred microliters of NADPH (0.12 mg/ml) was added, and the microcuvettes were shaken by inversion and incubated as described above for GSHpx. Then 6.1 GSSG was added, the cuvette was again shaken by inversion, and the preparation was read at 340 nm. The decrease in absorbance was recorded every 30 s for 5 min.

Glutathione transferase

GSHtf activity was determined according to the method of Warholm et al. (1985). One hundred microliters of GSH (0.3 mg/ml) was added to the volumes of sample and buffer noted above for the other enzyme determinations. The mixture was shaken by inversion and incubated at 37 °C for 3 min. Then 2 mg/ml 1-chloro-2,4-dinitrobenzene was added, the mixture was shaken by inversion, and the preparation was read at 340 nm as for the other enzyme activities.

Statistical methods

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

Results

Zoometric and blood parameters

As shown in Table 1, there were no significant differences in body weight, weight of the internal organs or hematological parameters except for glycemia in diabetic animals in comparison to healthy control rats. None of the three treatments compared here significantly modified glycemia values.

![Graph](image)

Fig. 5. Plasmatic nitrite+nitrate (NO$_2^-$+NO$_3^-$) concentration in normoglycemic rats (control) and diabetic rats (2 months’ duration) treated orally with saline, or with 2 mg/kg/day aspirin (ASA), 25 mg/kg/day alpha-tocopherol (AT), or both in combination. Each bar represents the mean±S.E.M. of 15 rats. *P<0.0001 with respect to control rats, **P<0.0001 and ***P<0.05 with respect to saline-treated diabetic rats.
Platelet parameters

Untreated diabetic animals had the highest maximal intensity of platelet aggregation (52.0%, in comparison to normoglycemic animals). Treatment with ASA reduced maximal intensity of platelet aggregation by 71.6% in comparison to animals that were made diabetic but not treated. The reduction obtained with alpha-tocopherol alone was 30.9%. The combination of both drugs led to an 88.9% inhibition of platelet aggregation compared to rats that were made diabetic but not treated (Fig. 1).

Platelet thromboxane B2 production increased by 115% in diabetic but untreated animals, in comparison to normoglycemic rats. Treatment with ASA reduced thromboxane synthesis by 84.66%, and alpha-tocopherol reduced thromboxane synthesis by 58.6%. The combination of both drugs reduced thromboxane production by 90.8% (Fig. 2).

Vascular parameters

6-Keto-PGF1α production was 66.2% lower in untreated animals than in normoglycemic rats. Acetylsalicylic acid reduced the vascular synthesis of prostacyclin by 23.58%, and alpha-tocopherol increased synthesis by 30.5%. The combination of both drugs increased prostacyclin synthesis by 162% in comparison to the group of rats made diabetic but not treated (Fig. 3).

We calculated the TxB2/6-keto-PGF1α ratio as a reflection of overall thromboxane and prostacyclin production (Fig. 4). In untreated diabetic animals had the highest maximal intensity of platelet aggregation (52.0%, in comparison to normoglycemic animals). Treatment with ASA reduced maximal intensity of platelet aggregation by 71.6% in comparison to animals that were made diabetic but not treated. The reduction obtained with alpha-tocopherol alone was 30.9%. The combination of both drugs led to an 88.9% inhibition of platelet aggregation compared to rats that were made diabetic but not treated (Fig. 1).

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Tissue oxidative stress parameters

Experimental diabetes led to a significant increase in lipid peroxide production (TBARS) and to a reduction in the main antioxidant system (glutathione pathway). The concentration of reduced glutathione (GSH) was decreased in all tissues analyzed here (Table 2). Enzyme activities related with the glutathione system are shown in Table 3. The most notable changes were the decrease in glutathione peroxidase and glutathione transferase activity in all tissues.

Pharmacological treatment (Table 2) led in all groups to a decrease in TBARS, an effect that was greater with alpha-tocopherol alone that with ASA alone. In the glutathione system, ASA had little effect on reduced glutathione (GSH) except in red blood cells, whereas its effect was greater when it was associated with alpha-tocopherol. Oxidized glutathione was practically normoglycemic animals this ratio was significantly lower than in untreated diabetic rats; both ASA and alpha-tocopherol reduced this ratio, but the higher reduction was observed when the two drugs were associated.

Plasma concentration of nitrates+nitrites, used as an indicator of total nitric oxide production, was 41.6% lower in untreated animals than in normoglycemic animals. Acetylsalicylic acid increased production by 125%, alpha-tocopherol increased production by 142%, and the combination of both drugs increased nitrates+nitrites by 241% in comparison to untreated animals (Fig. 5).

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>DR</th>
<th>ASA</th>
<th>AT</th>
<th>ASA + AT</th>
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<td>TBARS</td>
<td>0.93±0.08</td>
<td>1.29±0.09**</td>
<td>1.17±0.07</td>
<td>1.11±0.05‡‡</td>
<td>0.70±0.05§</td>
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<tr>
<td>GSH</td>
<td>42.73±1.14</td>
<td>30.1±1.85†</td>
<td>28.33±0.63</td>
<td>30.71±0.99</td>
<td>31.2±0.88</td>
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<td>GSSG</td>
<td>4.48±0.22</td>
<td>3.26±0.07*</td>
<td>3.99±0.15‡</td>
<td>2.81±0.24*</td>
<td>2.21±0.124‡‡</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.71±0.04</td>
<td>0.93±0.05*</td>
<td>0.82±0.03</td>
<td>0.57±0.039††</td>
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<tr>
<td>GSH</td>
<td>14.61±0.35</td>
<td>12.04±0.52**</td>
<td>12.94±0.42</td>
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<td>13.51±0.49††</td>
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<tr>
<td>GSSG</td>
<td>3.24±0.13</td>
<td>1.92±0.16††</td>
<td>0.79±0.04‡§</td>
<td>0.81±0.06§§</td>
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<td>Brain</td>
<td>0.59±0.04</td>
<td>3.59±0.56††</td>
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<td>0.61±0.04§§</td>
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<tr>
<td>GSH</td>
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<td>Red blood cells</td>
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<td>1.29±0.09††</td>
<td>0.91±0.03†</td>
<td>0.51±0.05§§</td>
<td>0.51±0.05§§</td>
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<tr>
<td>GSH</td>
<td>5.29±0.27</td>
<td>2.49±0.13**</td>
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<td>3.87±0.26§</td>
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<td>GSSG</td>
<td>0.90±0.09</td>
<td>0.61±0.05**</td>
<td>0.58±0.03</td>
<td>1.10±0.11§§</td>
<td>1.18±0.13§§</td>
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<tr>
<td>Plasma</td>
<td>0.69±0.06</td>
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<tr>
<td>GSSG</td>
<td>1.06±0.08</td>
<td>1.22±0.13*</td>
<td>1.79±0.19††</td>
<td>1.04±0.09b</td>
<td>1.14±0.16b</td>
</tr>
</tbody>
</table>

In each group n=15 rats. *P<0.05, **P<0.01, ††P<0.001, with respect to NDR. ‡P<0.05, ‡‡P<0.01, §§P<0.001, with respect to DR. aP<0.05 with respect to AT and ASA but nonsignificant for AT versus ASA, bP<0.05 with respect to ASA but nonsignificant for AT versus AS + AT, cP<0.05 with respect to AT and ASA.
versus ASA, b 2001). It should be noted that blood glucose levels were well controlled in the animals or human participants involved in these studies. In the present study the decrease in total glutathione was obtained with the same experimental model (De La Cruz et al., 1997; Ishii et al., 1996; Moreno et al., 1995; Vallejo et al., 2000). It thus seems clear that animals with diabetes of 2 months’ duration show changes in the platelet/endothelium interaction that may account for at least part of the higher incidence of thrombotic phenomena described in persons with diabetes mellitus.

Our results for the parameters relating to changes in oxidative stress are consistent with the findings of earlier studies that found higher levels of lipid peroxides in diabetes, both in animal models (Reagan et al., 2000; Sun et al., 1999) and in human patients with type 1 (Vessby et al., 2002) or type 2 diabetes (Aydin et al., 2001). Our findings also indicate deficient glutathione storage in all tissues analyzed here. However, some authors failed to find significant changes in glutathione levels in the rat brain (Genet et al., 2002; Ramanathan et al., 1999; Santos et al., 2000) or in plasma or erythrocyte levels of glutathione in humans (Aydin et al., 2001). It should be noted that blood glucose levels were well controlled in the animals or human participants involved in these studies. In the present study the decrease in total glutathione was accompanied by a decrease in reduced glutathione levels in all tissues. Depletion of the main reserves of antioxidant substances in tissues may be a result of greater oxidative damage and diminished activity of the mechanism of GSH recovery in diabetes (Aydin et al., 2001; Baynes and Thorpe, 1994; Ceriello et al., 1996). This depletion may be related to a decrease in GSH transferase activity (Table 3), or to a possible deficit in the cellular supply of GSH to the tissues we studied.

The effect on platelet-related parameters of the acetylsalicylic acid–alpha-tocopherol combination tested in our experimental model of diabetes was similar to that reported in an earlier study from our laboratory of whole blood parameters in healthy volunteers (González-Correa et al., 2005), and to the results reported by others (Celestini et al., 2002) who analyzed the effect of this combination in platelet-rich plasma from healthy volunteers. Alpha-tocopherol alone had little effect on platelet aggregation, but the antiaggregant effect was potentiated when it was associated with ASA. The findings for the inhibition of platelet thromboxane production were similar; alpha-tocopherol alone had little effect, and when associated to ASA little enhancement in the inhibitory effect of aspirin was seen. This may be a result of the ability of ASA to potently and irreversibly block platelet cyclooxygenase, and of the lack of effect of alpha-tocopherol and its derivatives on other enzymes related to thromboxane synthesis, such as thromboxane synthase (Sheu et al., 1999).

As in an earlier report (González-Correa et al., 2005), our results show that alpha-tocopherol spares and can even increase prostacyclin levels, a result with interesting implications for a process such as diabetes mellitus, in which prostacyclin synthesis is inhibited in comparison to nondiabetic animals. This

<table>
<thead>
<tr>
<th>Tissues</th>
<th>NDR</th>
<th>DR</th>
<th>ASA</th>
<th>AT</th>
<th>ASA + AT</th>
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<td></td>
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<tr>
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<td>21.85±0.98‡</td>
<td>27.93±0.76§</td>
<td>26.30±0.88</td>
</tr>
<tr>
<td>GSHtf</td>
<td>89.92±1.70</td>
<td>54.36±3.30††</td>
<td>61.15±5.02†</td>
<td>63.43±3.05§</td>
<td>78.40±3.20§§</td>
</tr>
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<td>Brain</td>
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<tr>
<td>GSHpx</td>
<td>51.11±0.82</td>
<td>16.50±1.31† †</td>
<td>50.38±6.32§</td>
<td>55.57±5.44§§</td>
<td>63.90±2.66§§</td>
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<tr>
<td>GSSGrd</td>
<td>9.72±0.27</td>
<td>10.00±0.40</td>
<td>12.07±0.36‡</td>
<td>12.14±0.66†</td>
<td>13.80±0.67‡‡</td>
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<tr>
<td>GSHtf</td>
<td>33.09±1.34</td>
<td>23.42±1.00**</td>
<td>37.69±1.58† †</td>
<td>39.14±1.86‡‡</td>
<td>49.20±2.53§§</td>
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<td>Aorta</td>
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<tr>
<td>GSHpx</td>
<td>69.64±4.99</td>
<td>54.64±5.61*</td>
<td>84.46±3.08§§</td>
<td>82.93±3.80§§</td>
<td>67.67±4.38*‡</td>
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<tr>
<td>GSSGrd</td>
<td>15.01±0.25</td>
<td>8.57±0.21†</td>
<td>8.85±0.69</td>
<td>9.93±0.70</td>
<td>8.44±0.42</td>
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<tr>
<td>GSHtf</td>
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<td>6.09±0.62†</td>
<td>10.54±1.35§</td>
<td>9.14±0.79§</td>
<td>13.85±1.12§§</td>
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<td>Red blood cells</td>
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<tr>
<td>GSHpx</td>
<td>28.82±2.72</td>
<td>39.37±2.94*</td>
<td>30.26±0.69</td>
<td>27.40±0.43‡</td>
<td>29.99±0.28‡</td>
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<tr>
<td>GSSGrd</td>
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<td>45.44±0.82</td>
<td>22.61±1.13§§</td>
<td>10.82±0.82§§</td>
<td>13.77±0.60§§</td>
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<tr>
<td>GSHtf</td>
<td>93.73±4.18</td>
<td>72.56±6.47**</td>
<td>100±3.09§§</td>
<td>115±7.22§§</td>
<td>129±4.33§§§</td>
</tr>
</tbody>
</table>

Data are shown for different tissues from nondiabetic ras (NDR), untreated diabetic rats (DR) and for diabetic rats treated orally for 2 months with 2 mg/kg/day acetylsalicylic acid (ASA), 25 mg/kg/day alpha-tocopherol (AT), or both drugs together at the same doses. In each group n = 15 rats. *P < 0.05, **P < 0.01, †P < 0.001, ††P < 0.0001, with respect to NDR. †P < 0.05, ††P < 0.01, §P < 0.001, §§P < 0.0001, with respect to DR. *P < 0.05 with respect to AT and ASA but nonsignificant for AT versus ASA, §P < 0.05 with respect to ASA but nonsignificant for AT versus ASA + AT, ‡P < 0.05 with respect to AT and ASA.

Discussion

In our experimental model of diabetes, we observed hyperactivity in platelet aggregation, increased thromboxane production, and an evident decrease in prostacyclin and nitric oxide production. These findings are consistent with earlier results obtained with the same experimental model (De La Cruz et al., 1997; Ishii et al., 1996; Moreno et al., 1995; Vallejo et al., 2000). It thus seems clear that animals with diabetes of 2 months’ duration show changes in the platelet/endothelium interaction that may account for at least part of the higher incidence of thrombotic phenomena described in persons with diabetes mellitus.
may reflect the fact that alpha-tocopherol inhibits lipid peroxidation, which in turn prevents lipid peroxides from curtailling prostacyclin synthase activity in the vascular wall (Grzyglewski and Szczeklik, 1978). In addition, our results show that the combination of ASA and alpha-tocopherol in our experimental model offsets the inhibitory effect of ASA.

Our findings thus show that the combination of ASA with alpha-tocopherol improves the severely altered thromboxane/prostacyclin interactions (i.e., the prothrombotic/antithrombotic balance) in rats made diabetic. The improved balance is a result of increased thromboxane synthesis and decreased prostacyclin synthesis. The combination of ASA with alpha-tocopherol reduced the thromboxane/prostacyclin ratio to values even lower than in the group of normoglycemic rats, and thus appears to be potentially useful as a prophylactic treatment.

Associating alpha-tocopherol to ASA nearly doubled the effect of aspirin alone on vascular NO production, which reached values even higher than those in normoglycemic control animals. Alpha-tocopherol reduced oxidative stress probably by lengthening the half-life of NO (Li et al., 2001), whose synthesis by platelets is stimulated by ASA and alpha-tocopherol (Keaney et al., 1999). The combination of these two drugs thus helped to restore optimal levels of endogenous antithrombotic factors altered by diabetes, i.e., prostacyclin and NO.

These drugs also modify the biochemical parameters of tissue oxidative stress in diabetic animals. The ability of the combination of ASA with alpha-tocopherol to inhibit peroxidation is not markedly greater than that of either drug alone, possibly because the blocking action of each drug separately is sufficient to fully inhibit peroxidation. However, their combination ensures a potent reduction in tissue levels of lipid peroxides, which are clearly increased in our experimental model of diabetes mellitus. The greater effect in brain and vascular tissues was a logical finding since the brain is the organ with the highest membrane lipid content, and arteries have the highest polyunsaturated fatty acid content (the fatty acids most susceptible to oxidation) of all tissues. The effect of alpha-tocopherol was expected in view of its mechanism of action (Amoroso et al., 1999; Osakada et al., 2003). Earlier work in our laboratory showed that ASA and its metabolite salicylic acid had a neuroprotective effect which acted via its antioxidant effect in brain tissue (De La Cruz et al., 2004; Guerrero et al., 2004).

Acetylsalicylic acid had little discernible effect on reduced glutathione content in the tissues we analyzed, except for red blood cells. This is consistent with the findings of Kikova et al. (1995). In addition, the effect of alpha-tocopherol in different tissues from diabetic animals has been reported before (Baydas et al., 2002; Kedziora-Komatowska et al., 2003). What is novel about the present study is our finding that the combination of ASA and alpha-tocopherol potentiated the increase in reduced glutathione levels, especially in vascular tissue. This effect might enhance the antithrombotic effect of ASA, as any antioxidant effect—whether it inhibits peroxidation or stimulates the glutathione system in cardiovascular tissues—implies increased availability of biochemical factors with antithrombotic action, such as prostacyclin and NO. Moreover, in vascular tissues this combination increased glutathione transferase activity, an effect that reflected a greater capacity for recovery of the glutathione system in the face of oxidative stress. In general, glutathione peroxidase activity and the percentage of glutathione in oxidized form in the tissues we analyzed were not changed by treatment, and in some tissues these values were actually lower in treated animals because the combination of the two drugs decreased oxidative damage, possibly making additional measures to protect tissues from oxidative stress redundant.

Our results show unequivocally that in animals with type-1-like diabetes, two key elements of the ischemic process were altered: platelet–vessel wall interactions were enhanced, and increased tissue oxidative stress could make this tissue more susceptible to ischemic damage. This justifies the potential usefulness of studying the combination of an antiplatelet drug (acetylsalicylic acid) (De La Cruz et al., 1987) and a standard antioxidant (alpha-tocopherol) (Villalobos et al., 1994).

These results support the potential usefulness of this combination to protect tissues from thrombotic and ischemic phenomena in diabetes mellitus, a disease in which the incidence and severity of these events are high. Moreover, this possible use of the alpha-tocopherol–ASA combination could be extended to diabetic microangiopathy, in which oxidative stress is the focus of the endothelial dysfunction and platelet hyperactivity is the source of microthrombotic ischemic complications.

We conclude that in our experimental model of diabetes mellitus, the combination of acetylsalicylic acid with alpha-tocopherol modified interactions between platelets and the vascular wall, and phenomena involved in tissue oxidative stress.

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References


