Microvascular Permeability With Sulfonylureas in Normal and Diabetic Hamsters

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The hamster cheek pouch is an experimental model in which quantitative studies of macromolecular permeability can be made by direct observation of extravasated fluorescein isothiocyanate (FITC)-dextran (leaks). The advantage of this model is that simultaneous light and fluorescent-light microscopy observations can be performed with instantaneous correlations between the site of FITC-dextran extravasation and the vessel morphology. The aims of our study were to compare, using the cheek pouch preparation, the effects of two sulfonylureas, gliclazide and glibenclamide, on the macromolecular permeability increase induced by histamine using control (normoglycemic) hamsters. In these studies, FITC-labeled dextran 150,000 daltons was administered intravenously and quantified by UV-light microscopy, and the drugs used were applied topically at therapeutic concentrations. Gliclazide and glibenclamide dose-dependently decreased the macromolecular permeability increase induced by histamine. This effect of gliclazide could be blocked by nifedipine (Ca^{2+} channel blocker) and not by diazoxide (K^+ channel opener), whereas for glibenclamide it could be blocked by diazoxide and not by nifedipine. To better characterize the antioxidant capacity of gliclazide and glibenclamide, their effect on the macromolecular permeability increase induced by ischemia/reperfusion was also compared with the effect of vitamin C in diabetic hamsters (glycemia > 240 mg/dL). Total ischemia of the preparation was obtained with a cuff placed around the neck of the everted pouch. Diabetes was induced by three intraperitoneal injections of streptozotocin 50 mg/kg/d in 3 days. In diabetic hamsters during ischemia/reperfusion, gliclazide was more effective in inhibiting the macromolecular permeability increase than glibenclamide (136.0 ± 5.8 leaks/cm² for placebo; 68.0 ± 2.9 for 1.2×10^{-6} mol/L gliclazide; 55.3 ± 3.5 for 1.2×10^{-5} mol/L gliclazide; 89.2 ± 5.7 for 8×10^{-8} mol/L glibenclamide; 107.0 ± 3.8 for 8×10^{-7} mol/L glibenclamide; 56.7 ± 3.4 for 10^{-6} mol/L vitamin C; and 20.5 ± 0.6 for 10^{-5} mol/L vitamin C). Our results suggest that (1) the inhibition of the permeability increase induced by histamine elicited by gliclazide may be mediated by Ca^{2+} channels, while that of glibenclamide may be mediated by K^+ channels, and (2) gliclazide appears to have an antioxidant capacity in ischemia/reperfusion injury similar to that of 10^{-6} mol/L vitamin C. Improvement in the microcirculation was independent of the hypoglycemic properties of the drug.

THE HAMSTER CHEEK POUCH is an experimental model in which quantitative studies of macromolecular permeability can be made by direct observation of extravasated fluorescein isothiocyanate (FITC)-dextran. The advantage of this model is that simultaneous light and fluorescent-light microscopy observations can be performed with instantaneous correlation between the site of FITC-dextran extravasation and the vessel morphology. Microvessel permeability can be increased by histamine, bradykinin, etc., treatment in control or diabetic animals.

The ability of organs to tolerate ischemia is dependent on the nature of the tissue involved, and the damage induced appears to be compounded during reperfusion. The oxygenated blood that infiltrates ischemic tissue during reperfusion is rich in reactive oxygen metabolites, and it has been suggested that these contribute to the initiation of injury.[1-3] The postischemic injury is associated with metabolic and morphological changes that are more or less severe depending on the duration of the ischemic period. Reperfusion of ischemic tissue could be followed by (1) a macromolecular permeability increase, (2) an increased number of leukocytes adhering to the postcapillary venules, and (3) a temporary increase in blood flow, so-called reactive hyperemia.[4] Several different experimental models have been devised to study the effect of ischemia/reperfusion in vivo. However, not many models offer the ability to observe acute changes at the microcirculatory level. The thin distal part of the hamster cheek pouch is highly vascularized and well suited for intravital microscopy studies of postischemic events in the microcirculation.[5]

The purposes of our study using the hamster cheek pouch preparation were to determine the effects of two sulfonylureas (gliclazide and glibenclamide) applied topically on the macromolecular permeability increase induced by histamine. The influence of a K^+ channel agonist (diazoxide) and a Ca^{2+} channel antagonist (nifedipine) was also studied. To avoid the effects of hyperglycemia, these experiments were performed using control hamsters. We also determined the effects of gliclazide and glibenclamide on the macromolecular permeability increase induced by ischemia/reperfusion in severely diabetic hamsters (glycemia > 240 mg/dL) subjected to 30 minutes of total ischemia. To better characterize the possible antioxidant effect of the two sulfonylureas on the macromolecular permeability increase induced by ischemia/reperfusion, we compared the effects of these drugs with the effects elicited by vitamin C.

MATERIALS AND METHODS

Experiments were performed on 54 control normoglycemic and 54 diabetic male hamsters (*Mesocricetus auratus*) divided into groups of six animals each aged 7 to 10 weeks and weighing approximately 100 g. Diabetes was induced by three intraperitoneal injections of streptozotocin 50 mg/kg body weight/dose dissolved in citrate buffer.[6] The diabetic animals did not receive any specific treatment for diabetes.

Surgical Procedures

On the day of the experiment, which for diabetic animals was day 31 after the onset of diabetes, anesthesia was induced by intraperitoneal

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injection of 0.1 to 0.2 mL sodium pentobarbital 60 mg/mL (Pentobarbital sodique; Sanofi, Paris, France) and maintained with α-chloralose 100 mg/kg (1,2-*O*-(2,2,2-trichloroethylidene)-α-D-glucofuranose; Merck, Darmstaadt, Germany) administered through the femoral vein. The femoral artery was also cannulated for pressure measurements. Throughout the operation and subsequent experiment, the temperature of the animals was kept at 37.5°C with a heating pad controlled by a rectal thermistor. A tracheal tube was inserted to facilitate spontaneous breathing.

The hamster was placed on a microscope stage similar to that described by Duling[7] with minor modifications. The cheek pouch was gently everted and pinned with four to five needles into a circular well filled with silicone rubber to provide a plane bottom layer, thus avoiding stretching of the tissue but preventing shrinkage. In this position, the pouch was submerged in a superfusion solution that continuously flushed the pool of the microscope stage. Before the pouch was pinned, large arterioles and venules were located with the aid of a Zeiss (Muttich, Germany) binocular stereomicroscope.

Fashioning of a single-layer preparation started with incision of the upper layer to swing a triangular flap to one side. The exposed area was dissected at 10× to 16× magnification under the stereomicroscope, and the fibrous, almost avascular, connective tissue covering the vessels was removed with ophthalmic instruments. The dissected part of the pouch was 125 to 150 μm thick. Dissected pouches with petechial formations or those without blood flow in all vessels were discarded.

Solutions

The superfusion solution was a HEPES-supported HCO_3^--buffered saline solution (composition in mmol/L: NaCl 110.0, KCl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 18.0, HEPES 15.39, and HEPES Na^+ salt 14.61) with temperature maintained at 36.5°C, and the superfusion rate was 6 mL/min. The pH was set at 7.40 by bubbling the solutions continuously with 5% CO_2 in 95% N_2.

Drugs

Solutions containing drugs (diazoxide, Sigma Chemical, St Louis, MO; FITC-dextran 150,000 daltons, BioflorB, Uppsala, Sweden; glibenclamide, Sigma Chemical; gliclazide, Servier Laboratories, Courbevoie, France; and histamine, nifedipine, streptozotocin, and vitamin C, Sigma Chemical) were freshly prepared for each experiment.

Intravital Microscopy

Observations were made with a Leitz Ortholux microscope with a 3.5× objective and 10× oculars. The light source was a 100-W mercury DC lamp (Irem [Berlin, Germany] model EL-XH5P/L). The specific filters used for observations in fluorescent light (Leitz [Westplatz, Germany] BG12, BG38, GG455, and KP409) were positioned between the light source and the condensor to give light for optimal excitation at 490 nm of the FITC-dextran. A barrier filter (K530) was placed between the objective and the eyepiece. The total observed area of the pouch was roughly circular and was estimated from the mean of two diameters, proximal to distal and left side to right side, measured with a calibrated scale at 35× magnification.

Permeability Studies

Thirty minutes after completion of the preparative procedure, FITC-dextran with a degree of substitution of two FITC molecules per 1,000 glucose molecules in the polysaccharide chain was administered at a dose of 25 mg/100 g as an intravenous injection of a 5% solution in 0.9% saline.[8]

Observations of the number of leakage sites (leaks) were made by manually scanning the total observation area twice at 35× magnification at selected intervals, ie, at 2, 5 (maximum number of leaks; Figs 1 and 2), 7, 10, 15, 20, and 30 minutes after beginning each topical

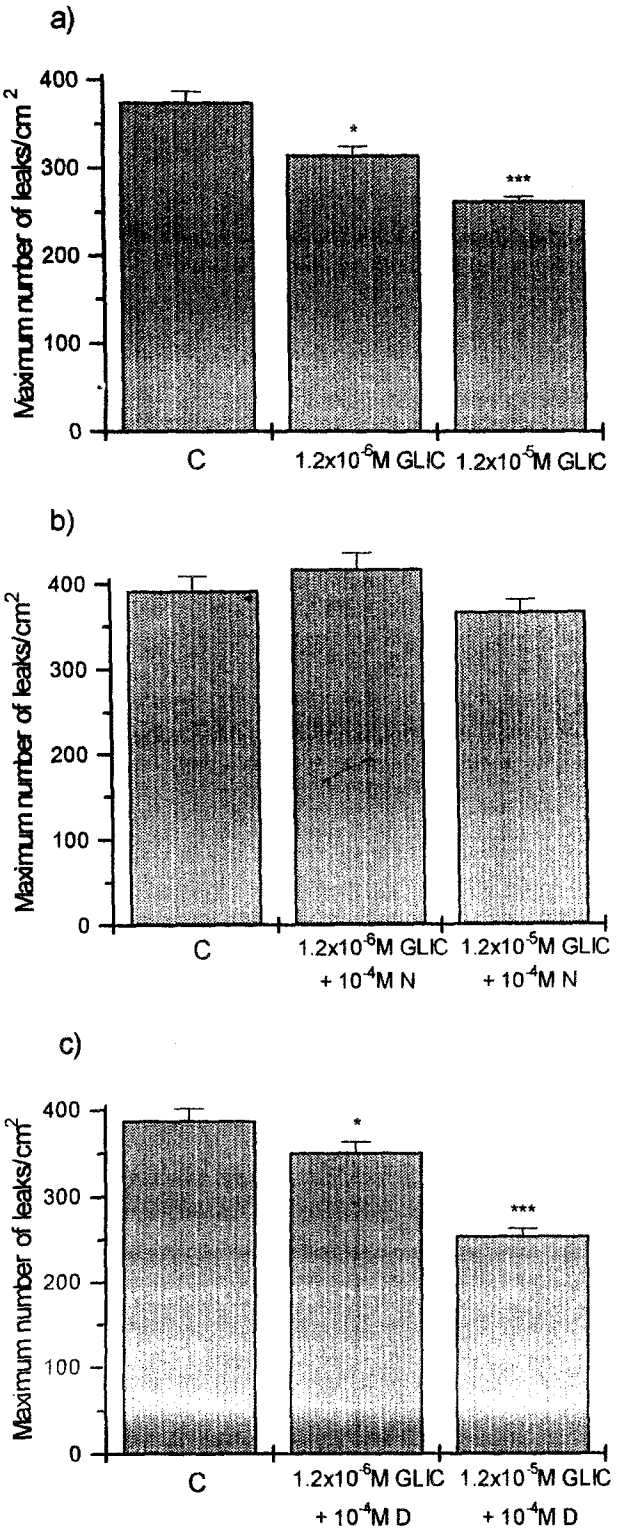

Fig 1. Mean ± SEM values for maximal number of venular leaky sites per cm² recorded 5 minutes after beginning histamine stimulation in control animals (a) combined with gliclazide (GLIC), (b) combined with nifedipine (N) + GLIC, and (c) combined with diazoxide (D) + GLIC (6 animals in each group). Significantly different from control: *$P < .05$, **$P < .01$, and ***$P < .001$.

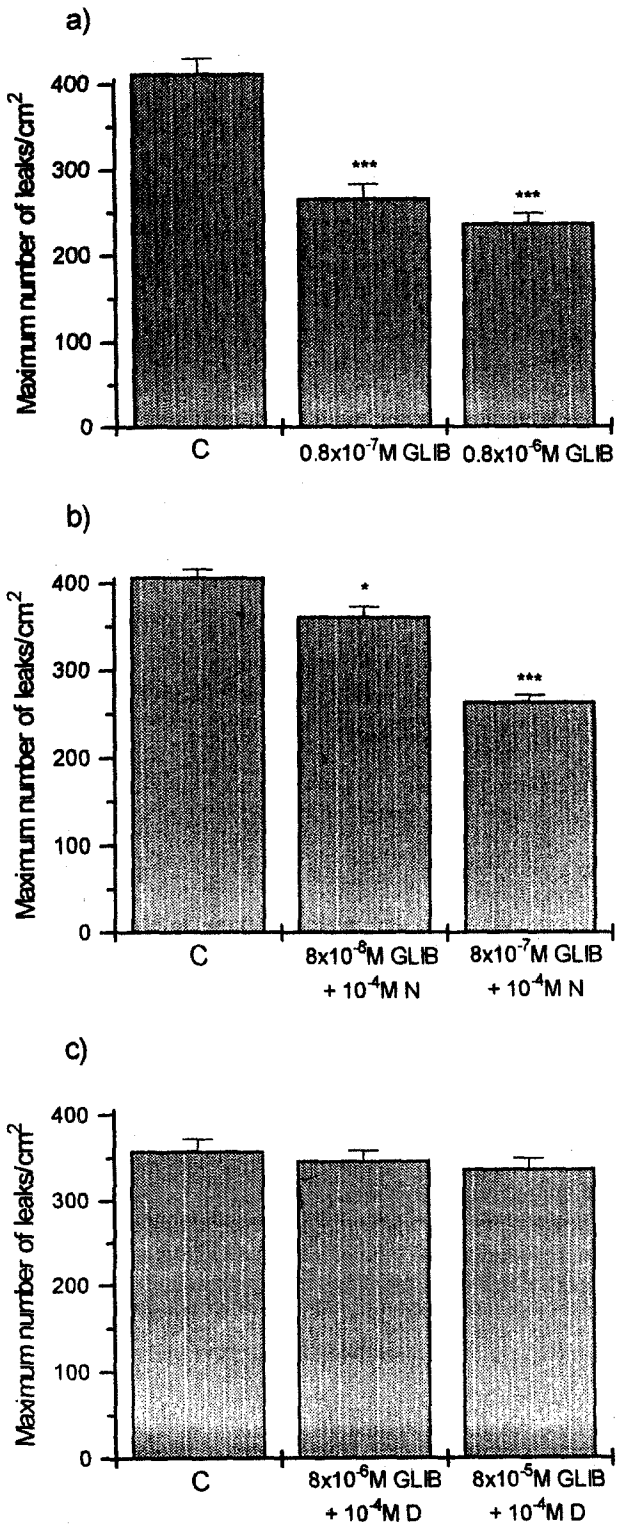

Fig 2. Mean ± SEM values for maximal number of venular leaky sites per cm² recorded 5 minutes after beginning histamine stimulation in control animals (a) combined with glibenclamide (GLIB), (b) combined with nifedipine (N) + GLIB, and (c) combined with diazoxide (D) + GLIB (6 animals in each group). Significantly different from control: *$P < .05$, **$P < .01$, and ***$P < .001$.

application (duration, 5 minutes) of histamine (final concentration, 2 μmol/L). The fluorescent spots formed at leakage sites could be traced when they reached a certain minimal size and fluorescent intensity. Each was classified as a leakage site when the diameter was greater than 100 μm. The number of leakage sites is reported per centimeter squared. All hamsters with the prepared area showing spontaneous nonfading leaks or greater than 10 fading leaks during the first 30-minute control period after FITC-dextran were discarded. Each hamster cheek pouch preparation was subjected to three to four local applications of histamine with a 1-hour interval between each application.

The concentrations of glibenclamide (8×10^{-8} and 8×10^{-7} mol/L) and gliclazide (1.2×10^{-6} and 1.2×10^{-5} mol/L) used were the same as found in the plasma of treated type II diabetic patients.[9-11]

Ischemia/Reperfusion

Local ischemia of the cheek pouch was obtained by a cuff made of thin latex tubing that was mounted around the neck of the everted pouch where it exits the mouth of the hamster.[5] The placement of the cuff can be chosen without any visible interference with local blood flow. The intratubular pressure can be rapidly increased by air compression using a syringe, and also can be rapidly decreased at evacuation. An intratubular pressure of 200 to 220 mm Hg in the cuff resulted in a complete arrest of microvascular blood flow, which eventually returned to a level similar to that observed before occlusion.

To quantify the macromolecular permeability increase, again 30 minutes after completion of the preparative procedure, FITC-dextran is given intravenously as described previously. The number of leaks are counted before and immediately after the ischemic period and every 10 minutes thereafter for 1 hour. The maximum number of leaks occured 10 min after the onset of reperfusion and this was the value used on Figs 3 and 4. All hamsters with the prepared area showing spontaneous nonfading leaks or greater than 10 fading leaks during the first 30-min control period, after FITC-dextran is given, were discarded.

Again, the concentrations of glibenclamide (8×10^{-8} and 8×10^{-7} mol/L) and gliclazide (1.2×10^{-6} and 1.2×10^{-5} mol/L) used were the same found in the plasma of treated type II diabetic patients.[9-11]

The results are presented as the mean ± SEM, unless otherwise noted. Statistical significance was determined by Student's *t* test, and *P* less than .05 was considered significant.

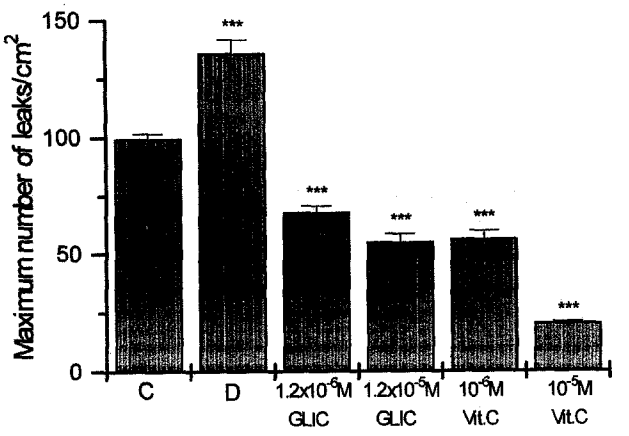

Fig 3. Mean ± SEM values for maximal number of venular leaky sites per cm² recorded 10 minutes after the onset of reperfusion after 30 minutes of total ischemia in control (C) and diabetic (D) animals, with further addition of gliclazide (GLIC) and vitamin C. Significantly different from control: *$P < .05$, **$P < .01$, and ***$P < .001$.

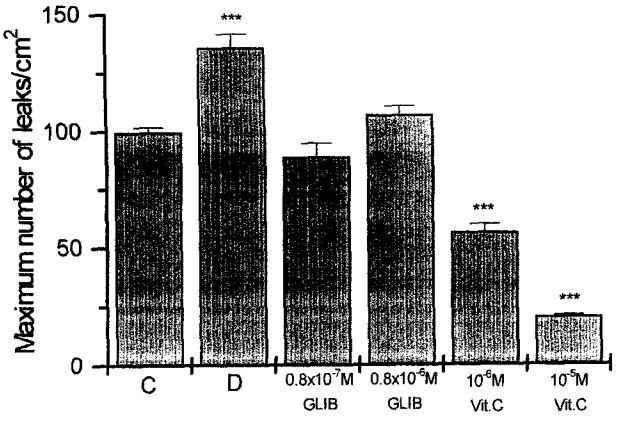

Fig 4. Mean ± SEM values for maximal number of venular leaky sites per cm² recorded 10 minutes after the onset of reperfusion after 30 minutes of total ischemia in control (C) and diabetic (D) animals, with further addition of glibenclamide (GLIB) and vitamin C. Significantly different from control: *$P < .05$, **$P < .01$, and ***$P < .001$.

RESULTS

No effect on mean arterial pressure could be detected in any of the groups studied (range, 95 to 105 mm Hg).

Permeability Measurements (control, normoglycemic hamsters)

The values reported represent the maximum number per centimeter squared, which occurred at 5 minutes after the beginning of each topical application of histamine. In the absence of any further addition to the superfusion solution, the four applications of histamine elicited, in sequential order, 380.5 ± 22.3, 381.3 ± 25.2, 361.2 ± 23.1, and 348.3 ± 23.2 leaks/cm². Addition of different concentrations of diazoxide (K^+ channel opener) did not significantly change the macromolecular permeability increase induced by histamine: 374.7 ± 17.4 for histamine only, 387.7 ± 15.7 for histamine + 10^{-8} mol/L diazoxide, 351.5 ± 11.9 for histamine + 10^{-6} mol/L diazoxide, and 310.3 ± 14.5 for histamine + 10^{-4} mol/L diazoxide. Addition of different concentrations of nifedipine (Ca^{2+} channel blocker) inhibited, in a dose-dependent fashion, the macromolecular permeability increase induced by histamine: 429.5 ± 19.1 for histamine only, 397.3 ± 19.1 for histamine + 10^{-8} mol/L nifedipine, 298.8 ± 17.5 for histamine + 10^{-6} mol/L nifedipine, and 239.8 ± 8.5 for histamine + 10^{-4} mol/L nifedipine. Addition of different concentrations of gliclazide inhibited, in a dose-dependent fashion, the macromolecular permeability increase induced by histamine (375.3 ± 12.5; Fig 1a). Further addition of nifedipine totally blocked the effects obtained with gliclazide alone (Fig 1b). On the other hand, further addition of diazoxide did not significantly change the effects obtained with gliclazide alone (Fig 1c). Addition of different concentrations of glibenclamide inhibited, in a dose-dependent fashion, the macromolecular permeability increase induced by histamine (Fig 2a). Further addition of nifedipine did not significantly change the effects obtained with glibenclamide alone (Fig 2b). On the other hand, further addition of diazoxide totally blocked the effects obtained by glibenclamide alone (Fig 2c).

Ischemia/Reperfusion (severely diabetic hamsters with glycemia 270 to 380 mg/dL and insulinemia 0.0 to 0.6 μU/mL)

The studied hamsters lost 30% to 40% of their body weight from the induction of diabetes until the day of the experiment, ie, 31 days. The postischemic increase in macromolecular permeability was significantly increased in diabetic compared with control animals: 136.0 ± 5.8 leaks/cm² for diabetic and 99.7 ± 2.2 for control normoglycemic hamsters. Topical application of gliclazide significantly inhibited the postischemic increase in macromolecular permeability in a dose-dependent fashion: 68.0 ± 2.9 for 1.2×10^{-6} mol/L gliclazide and 55.3 ± 3.5 for 1.2×10^{-5} mol/L. Topical application of glibenclamide elicited a smaller effect on the postischemic increase in macromolecular permeability: 89.2 ± 5.7 for 8×10^{-8} mol/L glibenclamide and 107.0 ± 3.8 for 8×10^{-7} mol/L. The effect observed with 1.2×10^{-5} mol/L gliclazide was similar to the effect observed with 10^{-6} mol/L vitamin C: 56.7 ± 3.4 for 10^{-6} mol/L vitamin C and 20.5 ± 0.6 for 10^{-5} mol/L; Figs 3 and 4.

DISCUSSION

The main findings of our study are that (1) at therapeutic concentrations, gliclazide and glibenclamide inhibited the macromolecular permeability increase induced by histamine in a dose-dependent fashion and by different mechanisms; (2) diazoxide by itself did not affect the macromolecular permeability increase induced by histamine; (3) nifedipine inhibited the macromolecular permeability increase induced by histamine in a dose-dependent fashion; (4) the effect of glibenclamide could be blocked by diazoxide and not by nifedipine; (5) the effect of gliclazide could be blocked by nifedipine and not by diazoxide; (6) gliclazide elicited a more potent inhibition than glibenclamide of the postischemic increase in vascular permeability to macromolecules; and (7) the effect of gliclazide on the postischemic increase in macromolecular permeability was equivalent to 10^{-6} mol/L vitamin C.

Histamine stimulates endothelial cells directly through specific receptors. Electron photomicrographs show that the leaks occur between adjacent endothelial cells owing to separation of loose junctions in the postcapillary venules creating a gap approximately 1 μm in diameter.[12] As long as the exposure to histamine is 5 minutes or less, there is no tachyphylaxis, ie, repeated applications of a given dose, after the return to the control state, produce a similar increase in the number of leakage sites in a 3- to 4-hour period.[13] The mechanism by which histamine causes endothelial cell separation in postcapillary venules is not completely clear, but may involve actomyosin-like contractile filaments.[14]

Increased macromolecular permeability through widened gaps between contracted endothelial cells of postcapillary venules is an important event in inflammatory reactions and edema formation. Because no significant changes in mean arterial pressure could be detected between the groups, the driving forces for filtration and reabsorption at the microcirculatory level remained approximately constant, and these drugs may interfere in the mechanism of gap formation, in which calcium flux plays an important role.[15,16]

Microvascular deterioration in diabetes has been associated with increased blood flow, venular dilatation, basement mem-

brane thickening, increasing exudation of plasma proteins, decreased density of microvessels, edema formation, and decreased oxygen supply to the tissues.[17] Oxygen free radicals have long been suggested to be involved in the inflammatory process. More than a decade ago, it was proposed that they could also contribute to the ischemia/reperfusion-induced injuries.[18,19] Superoxide dismutases (SODs) are the main defense in the body against a superoxide attack. In the hamster cheek pouch, SOD inhibited the postischemic permeability increase, showing that superoxide radicals are involved in the ischemia/reperfusion-induced events in the hamster.[20] Previous studies have shown that the sulfonylureas, specifically gliclazide, might act as a free radical scavenger.[21-24] In diabetic and nondiabetic subjects, gliclazide and vitamin C inhibited low-density lipoprotein oxidation in vitro, and gliclazide in therapeutic concentrations was as effective as vitamin C in preventing it.[22] In diabetic patients, Kähler et al[25] showed that oxidative stress promoted the development of diabetes complications. However, the mechanisms for generation of reactive oxygen species and the nature of the oxidative damage in these patients have to be clarified in more detail. Our results with topical application of gliclazide in vivo strongly suggest that it could act as a free radical scavenger in ischemia/reperfusion also with an efficiency similar to that of vitamin C.

Since all substances were topically applied, no effects on glycemia or insulinemia could be detected.

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