L-Arginine does not reverse impaired agonist-induced increases in macromolecular efflux during diabetes mellitus

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Abstract

Objective: The first goal of this study was to determine the effect of diabetes mellitus on agonist-induced increases in venular macromolecular permeability of the hamster cheek pouch. The second goal was examine the role for an alteration in the availability of L-arginine to nitric oxide synthase in impaired agonist-induced increases in macromolecular permeability during diabetes. Methods: We used intravital fluorescent microscopy and fluorescein isothiocyanate dextran (FITC-dextran; mw = 70 K) to examine macromolecular extravasation from post-capillary venules in non-diabetic and diabetic (2 weeks after injection of streptozotocin) hamsters in response to histamine and substance P. Increases in extravasation of macromolecules were quantitated by counting the number of venular leaky sites and by calculating the clearance (ml/s × 10^-6) of FITC-dextran-70K. Results: In non-diabetic hamsters, histamine 1.0 and 5.0 μM and substance P 50 and 100 nM increased permeability of the cheek pouch microcirculation to FITC-dextran-70K. In contrast, histamine- and substance P-induced increases in macromolecular extravasation were markedly reduced in diabetic hamsters. Next, we investigated whether alterations in histamine- and substance P-induced increases in macromolecular extravasation were markedly reduced in diabetic hamsters. We found that L-arginine potentiated agonist-induced increases in non-diabetic hamsters, but did not alter responses in diabetic hamsters. Conclusion: These findings suggest that short-term diabetes mellitus alters agonist-induced alterations in microvascular permeability. The mechanism of altered microvascular permeability during diabetes mellitus does not appear to be related to an impaired availability of L-arginine.

Keywords: Histamine; Nitric oxide; Permeability; L-Arginine; Diabetes; Substance-P; Immunofluorescence; Hamster, cheek pouch

1. Introduction

Diabetes mellitus produces morphological and functional alterations of the macro- and microcirculations. Morphologically, diabetes mellitus produces thickening of the capillary basement membrane [1,2], a decrease in the density of microvessels [3] and endothelial cell degeneration [4]. Functionally, diabetes mellitus produces an increase in basal transport of large and small molecules [5–8] and impairs endothelium-dependent responses of large and small peripheral arteries [1,9–15]. Thus, morphological and functional alterations in the macro- and microcirculations appear to contribute to the pathogenesis of vascular dysfunction during diabetes mellitus.

Many inflammatory mediators, including histamine and substance P, produce an increase in permeability of the macro- and microcirculations [16–23]. We [24–26] and others [16,17,27–30] have shown that increases in vascular permeability in response to many inflammatory mediators (including histamine and substance P) are related to the synthesis/release of nitric oxide or a nitric oxide containing compound. Previous studies have suggested that changes in agonist-induced increases in vascular permeability are depressed during long- and short-term diabetes
mellitus [31–34]. However, cellular mechanisms which contribute to alterations in agonist-induced changes in vascular permeability during diabetes mellitus are not clear. Our first goal was to examine the effect of short-term (2 weeks in duration) diabetes mellitus on macromolecular permeability of the hamster cheek pouch microcirculation in response to inflammatory mediators (i.e., histamine and substance P). Our second goal was to determine whether impaired agonist-induced increases in permeability of the cheek pouch microcirculation during diabetes mellitus could be related to an alteration in the L-arginine/nitric oxide biosynthetic pathway. Thus, we examined whether exogenous application of L-arginine could restore impaired agonist-induced alterations in macromolecular transport during diabetes mellitus.

2. Methods

2.1. Induction of diabetes mellitus

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication Number 85-23, revised 1985). Male Golden Syrian hamsters (100–120 g body wt) were divided randomly into non-diabetic and diabetic groups. All hamsters had access to food and water ad libitum. One group of hamsters was injected with streptozotocin (50 mg/kg i.p. for 3 consecutive days) to induce diabetes [5,35]. The second group of hamsters (non-diabetic) was injected with vehicle (sodium citrate buffer). Blood samples, for measurement of blood glucose concentration, were obtained 5–7 days after injection of streptozotocin or vehicle (samples obtained from a tail stick), and on the day of the experiment (2 weeks post-streptozotocin-injection; sample obtained from femoral artery catheter). Blood glucose concentration was determined in all hamsters by using a Glucoscan Meter (Lifescan, Inc., Mountain View, CA). On the day of the experiment, blood glucose concentration was 118 ± 12 mg/dl in non-diabetic hamsters and 371 ± 12 mg/dl in diabetic hamsters (P < 0.05). Body weight was 127 ± 3 g in non-diabetic hamsters and 110 ± 4 g in diabetic hamsters (P < 0.05).

2.2. Preparation of animals

On the day of the experiment the hamsters were anesthetized (pentobarbital sodium, 60 mg/kg body wt, i.p.) and a tracheotomy was performed to facilitate spontaneous breathing. The left femoral vein was cannulated for the purpose of injecting the intravascular tracer [fluorescein isothiocyanate dextran (FITC-dextran; m.w. 70 000 Da)] and for injection of supplemental anesthesia (20–40 mg/kg/h). The left femoral artery was cannulated for the purpose of measuring arterial pressure, which remained constant during the experimental period (data not shown).

To visualize the microcirculation of the cheek pouch, we used a method that we have described previously [18,36–38]. Briefly, the cheek pouch was spread over a small plastic baseplate and an incision was made in the skin to expose the cheek pouch membrane. An avascular layer of connective tissue was excised to expose the microvessels of the cheek pouch. An upper chamber was then positioned over the baseplate to provide a reservoir for the suffusion fluid. This arrangement forms a triple-layered complex: the baseplate, the upper chamber, and the cheek pouch microcirculation exposed between these two plates.

The cheek pouch microcirculation was continuously suffused with a bicarbonated buffer (37 ± 1°C). The cheek pouch chamber also was connected via a 3-way valve to a pump which allowed for infusion of agonists into the suffusate. This method which we have used previously [18,36–38] allowed us to maintain a constant temperature and pH of the suffusion fluid during application of agonists.

2.3. Evaluation of microvascular permeability

Alterations in macromolecular transport were quantified by counting the number of microvascular leaky sites, which occurred exclusively around post-capillary venules [18,36–38]. The number of leaky sites (number per 0.11 cm²) was determined under control conditions (before application of agonists) and at 1, 3, 5, 7, 10, 15, 20 and 30 min during and following a 5 min application of histamine or substance P. The number of leaky sites observed in two microscopic fields (0.11 cm² per microscopic field) under control conditions and during application of agonists was averaged, and we report responses as the average of these two microscopic fields [25,26,38].

In some hamsters [non-diabetic (n = 4) and diabetic (n = 6)], we examined the clearance of FITC-dextran before and following application of histamine. Thus, in these experiments, the suffusion fluid was collected in glass test tubes at 5 min intervals throughout the experiment with the aid of a fraction collector (Micro-fractionator, Gilson Medical Electronics, Inc., Middleton, WI). In addition, arterial blood samples were collected in heparinized capillary tubes (50–70 µl volume) before and at various intervals following injection of FITC-dextran.

To quantitate the concentration of FITC-dextran in the plasma and suffusate, a standard curve for FITC-dextran concentration versus percentage emission was performed on a spectrophotofluorometer (Model 650-10LC; Perkin-Elmer Corp., Norwalk, CT). The standard was FITC-dextran that was prepared on a weight per volume basis. Using bicarbonated buffer as background, a standard curve was generated for each experiment, and each curve was subjected to linear regression analysis. The percent emis-
sion for unknown samples (plasma and suffusate) was measured on the spectrophotofluorometer and the concentration of FITC-dextran was calculated from the standard curve [18, 25, 37, 39]. Clearance of FITC-dextran was determined by calculating the ratio of suffusate (ng/ml) to plasma (mg/ml) concentration of FITC-dextran and multiplying this ratio by the suffusate flow rate (2 ml/min) [18, 25, 37, 39].

2.4. Experimental protocol

In the first series of experiments, we examined the effects of histamine and substance P on macromolecular transport in non-diabetic (n = 18 for histamine and n = 9 for substance P) and diabetic (n = 14 for histamine and n = 11 for substance P) hamsters. After insertion of the cheek pouch chamber, the preparation was allowed to equilibrate for 40–45 min. Then, successive doses of histamine (1.0 and 5.0 μM) or substance P (50 and 100 nM) were superfused over the cheek pouch microcirculation for 5 min. There was a 50–60 min recovery period between the application of each concentration of histamine or substance P and a 50–60 min recovery period between application of histamine or substance P. Application of histamine and substance P were randomized. The number of venular leaky sites and clearance of FITC-dextran (for histamine) were determined as described above.

In a second series of experiments, we examined the effects of exogenous application of L-arginine on macromolecular transport in response to histamine and substance P in non-diabetic (n = 5 for histamine and substance P) and diabetic (n = 5 for histamine and substance P) hamsters. After insertion of the cheek pouch chamber, the preparation was continuously superfused with a bicarbonated buffer containing L-arginine (100 μM). After a 40–45 min equilibration period, successive doses of histamine (1.0 and 5.0 μM) or substance P (50 and 100 nM) were superfused over the cheek pouch microcirculation for 5 min, as described above. The number of venular leaky sites before and following suffusion with histamine or substance P in non-diabetic and diabetic hamsters was determined as described above.

2.5. Statistical analysis

An unpaired t-test was used to compare responses to histamine and substance P between non-diabetic and diabetic hamsters. In addition, an unpaired t-test was used to compare responses in untreated hamsters and in hamsters in which we superfused exogenous L-arginine. A P-value of 0.05 was considered to be significant.

3. Results

3.1. Responses in non-diabetic hamsters

In non-diabetic hamsters, there were no leaky sites visible under control conditions prior to application of histamine or substance P. Topical application of histamine and substance P produced a dose-related increase in the number of venular leaky sites in non-diabetic hamsters during the experimental period (Fig. 1). Leaky sites occurred around post-capillary venules, reached a maximum within 5–7 min after starting suffusion of agonist and resolved during the recovery period. Application of histamine also increased the clearance of FITC-dextran from the cheek pouch microcirculation. Clearance of FITC-de-
xtran increased from 0.25 ml/s × 10⁻⁶ under control conditions, to 0.71 ml/s × 10⁻⁶ during application of 1.0 μM histamine and to 1.2 ml/sec × 10⁻⁶ during application of 5.0 μM histamine in non-diabetic hamsters (n = 4). Thus, there appears to be a relationship between an increase in venular leaky site formation and the transport of macromolecules across post-capillary venules.

3.2. Responses in diabetic hamsters

In diabetic hamsters, there were no leaky sites visible under control conditions (prior to application of histamine or substance P). In contrast to that observed in non-diabetic hamsters, topical application of histamine and substance P produced only a minimal increase in the number of venular leaky sites in diabetic hamsters during the experimental period (Fig. 2). The maximum number of leaky sites in response to histamine and substance P was significantly less in diabetic hamsters than that observed in non-diabetic hamsters (Fig. 3). Furthermore, the clearance of FITC-dextran during suffusion with histamine was significantly less in diabetic than non-diabetic hamsters. In diabetic hamsters, clearance of FITC-dextran increased from 0.15 ml/s × 10⁻⁶ under control conditions, to only 0.19 ml/s × 10⁻⁶ during application of 1.0 μM histamine and to only 0.2 ml/s × 10⁻⁶ during application of 5.0 μM histamine (n = 6; P < 0.05 versus clearance in non-diabetic hamsters).

Fig. 2. Profile of venular leaky site formation (number per 0.11 cm²) during application of histamine (1.0 and 5.0 μM; n = 14) and substance P (50 and 100 nM; n = 11) in diabetic hamsters. Histamine and substance P were applied for a 5 min period. Values are means ± s.e.

Fig. 3. Maximum number of venular leaky sites (number per 0.11 cm²) during application of histamine (1.0 and 5.0 μM) and substance P (50 and 100 nM) in non-diabetic (open bars) and diabetic (closed bars) hamsters. Values are means ± s.e.

Fig. 4. Maximum number of venular leaky sites (number per 0.11 cm²) during application of histamine (1.0 and 5.0 μM) in non-diabetic hamsters (n = 5) before (open bars) and after (hatched bars), and in diabetic hamsters (n = 5) before (closed bars) and after (hatched bars) topical application of L-arginine (100 μM). Values are means ± s.e. * P < 0.05 versus response in non-diabetic hamsters before application of L-arginine. † P < 0.05 versus response in non-diabetic hamsters after application of L-arginine.
The results of the present study suggest that diabetes mellitus impairs agonist-induced increases in venular permeability which are presumably related to an increased synthesis/release of nitric oxide or a nitric-oxide-containing compound. In addition, our findings suggest that the mechanism of impaired agonist-induced increases in macromolecular extravasation during diabetes mellitus is not related to an impaired availability of L-arginine by nitric oxide synthase.

3.3. Effects of L-arginine

In non-diabetic hamsters, superfusion of L-arginine (100 μM) did not produce venular leaky sites prior to application of histamine or substance P. However, L-arginine (100 μM) did potentiate venular leaky site formation in response to histamine (Fig. 4) and substance P (Fig. 5).

In diabetic hamsters, superfusion of L-arginine (100 μM) did not produce venular leaky sites prior to application of histamine or substance P. In contrast to that observed in non-diabetic hamsters, L-arginine (100 μM) did not alter venular leaky site formation in response to histamine (Fig. 4) or substance P (Fig. 5). The number of venular leaky sites produced with histamine or substance P in diabetic hamsters treated with L-arginine was similar to that produced in diabetic hamsters that were not treated with L-arginine (P > 0.05). In addition, the maximum increase in venular leaky site formation in diabetic hamsters treated with L-arginine following application of histamine or substance P was significantly less than that observed in non-diabetic hamsters treated with L-arginine (P < 0.05).

4. Discussion

The hamster cheek pouch is an extensively used model to study the effects of inflammatory mediators on macro- and microcirculation [18,26,36,37,40–42]. In the present study we used in vivo fluorescent microscopy to examine extravasation of FITC-dextran from venules contained within the microcirculation of the hamster cheek pouch. In addition, we calculated the clearance of FITC-dextran in order to verify that changes in leaky site formation during application of an inflammatory mediator produce an increase in the transport of macromolecules from post-capillary venules. Thus, these methods allowed us to quantify the location and magnitude of changes in macro-molecular transport during stimulation with inflammatory mediators in non-diabetic and diabetic hamsters. We and others have used the evaluation of formation of venular leaky sites and clearance of FITC-dextran as means to quantitate changes in microvascular transport [18,36–38,40–43].

We considered the possibility that inhibition of histamine- and substance P-induced increases in venular leaky sites during diabetes mellitus may be related to the effects of diabetes on vascular diameter, which may therefore affect venular driving pressure. However, we have shown previously that changes in vascular diameter and/or venular driving pressure do not account for venular leaky site formation following application of inflammatory mediators [25,37,38]. We found that leukotriene C4, which produces constriction of cheek pouch arterioles, also produces a marked increase in the formation of venular leaky sites [25]. In addition, topical application of isoproterenol, which produces dilatation of cheek pouch arterioles and a similar increase in venular pressure as bradykinin, does not increase the formation of venular leaky sites [37,38]. Thus, we suggest that inhibition of venular leaky sites in response to histamine and substance P in diabetic hamsters is probably not related to the effects of diabetes on vascular diameter and/or venular driving pressure.

4.1. Consideration of methods

4.2. Consideration of previous studies

Studies which have examined the role of nitric oxide in agonist-induced alterations of the macro- and microcirculations during diabetes mellitus have, for the most part, concentrated on vascular reactivity, and not microvascular permeability. In brief, these previous studies suggest that diabetes mellitus produces profound impairment of nitric oxide synthase-dependent relaxation of large and small blood vessels in animal models and humans [1,9–11,13–15,44–51]. Mechanisms which account for the effects of diabetes on reactivity of blood vessels include the production of a cyclo-oxygenase constrictor substance with subsequent activation of the prostaglandin H2/thromboxane A2 receptor[51–53], increased chronic production of oxygen...
radicals [54–58], increased production of advanced glycosylation end-products [59–63], increased levels of sorbitol [64–69], and/or a deficit in the availability of L-arginine to nitric oxide synthase [70,71]. The role of these cellular pathways in impaired agonist-induced increases in macromolecular permeability during diabetes is not known, but could conceivably be related to activation of one or more of these cellular pathways.

Several studies have examined the role of nitric oxide in agonist-induced increases in vascular permeability. One study reports that generation of nitric oxide inhibits agonist-induced increases in vascular permeability [72]. In contrast, other investigators [16,17,27–30] have suggested that the synthesis and release of nitric oxide contributes to increases in vascular permeability in response to various stimuli, including histamine and substance P. In addition, we [24–26] have shown that increases in the formation of venular leaky sites and clearance of FITC-dextran from the hamster cheek pouch in response to inflammatory mediators are related to the synthesis/release of nitric oxide or a nitric-oxide-containing compound. In light of findings which suggest that nitric oxide synthase-mediated responses of blood vessels are altered during diabetes, impaired agonist-induced responses of blood vessels during diabetes may be related to an alteration in the availability of L-arginine and the contribution of nitric oxide in agonist-induced increases in microvascular permeability, we reasoned that nitric oxide synthase-mediated increases in macromolecular transport may be altered during diabetes mellitus. In support of this concept, we found that nitric oxide synthase-mediated increases in macromolecular transport due to stimulation with histamine and substance P were profoundly impaired in diabetic compared to non-diabetic hamsters.

Previous studies also have examined the effects of diabetes mellitus on agonist-induced increases in vascular permeability [31–34]. Early studies by Garcia-Leme [31,34] report that edema formation in the paw of rats in response to several inflammatory agonists (histamine, serotonin and bradykinin) was impaired in long-term alloxan-induced diabetic rats. This alteration in agonist-induced increases in vascular permeability in diabetic rats could be restored by treatment with insulin [31,34]. In another study, Llorach et al. [33] found that increases in permeability of the cremaster muscle to carbon particles in response to histamine and serotonin was less in long-term alloxan-induced diabetic rats than in non-diabetic rats. Again, treatment with insulin restored impaired agonist-induced increases in permeability in diabetic rats to that observed in non-diabetic rats. A recent study [32] suggests that the permeability of the rat cremaster muscle to FITC-albumin in response to serotonin was impaired during short-term (2 weeks) diabetes. The mechanism for the alteration in agonist-induced increases in macromolecular transport during short-term diabetes, however, was not investigated in this previous study [32]. In addition to examining the effect of diabetes on agonist-induced increases in macromolecular transport, investigators have examined a role for increased circulating levels of inflammatory mediators during diabetes mellitus [73,74]. These studies [73,74] have suggested that endogenous levels of histamine may be increased during diabetes mellitus (humans and rats) and may account for elevated basal levels of microvascular permeability observed during diabetes. Furthermore, it is possible that increased endogenous levels of inflammatory mediators (i.e., histamine) could produce downregulation of histamine receptors and thus could influence histamine-induced increases in macromolecular transport during diabetes.

In the present study, we did not find an increase in basal levels of leaky sites and/or clearance of FITC-dextran in diabetic hamsters. This may be related to the duration of diabetes and/or the size of the dextran molecule used to evaluate permeability. Thus, it is not clear whether downregulation of receptors for inflammatory mediators can account for impaired agonist-induced increases in macromolecular transport during diabetes. Our findings of impaired histamine- and substance P-induced increases in macromolecular transport are similar to those reported by other investigators [31–34]. However, the findings of the present study extend that of previous studies by examining a potential role for an alteration in the availability of L-arginine to nitric oxide synthase in impaired agonist-induced increases in macromolecular permeability during diabetes mellitus. We found that application of L-arginine potentiated histamine- and substance P-induced increases in macromolecular transport in non-diabetic hamsters, but not in diabetic hamsters. This finding suggests that impaired nitric oxide synthase-mediated increases in macromolecular transport in diabetic hamsters are not related to an impaired availability of L-arginine to nitric oxide synthase. Thus, it appears that mechanisms which contribute to impaired agonist-induced increases in microvascular permeability during diabetes are related to alterations in other cellular events including a direct alteration in the enzyme, nitric oxide synthase, an alteration in the L-arginine transporter system, increased production of oxygen radicals to inactivate nitric oxide, increased production of glycosylation end-products, and/or increased levels of sorbitol.

### 4.3. Conclusion

Short-term diabetes mellitus alters nitric oxide synthase mediated increases in venular macromolecular transport in the hamster cheek pouch. The mechanism for impaired nitric oxide synthase-mediated increases in macromolecular transport during diabetes mellitus does not appear to be related to an alteration in the availability of L-arginine since exogenous L-arginine did not restore the permeability characteristics in response to histamine and substance P in diabetic hamsters. Our findings suggest that diabetes mellitus, in addition to producing alterations in arterial and
arteriolar reactivity, produces profound alterations in the permeability characteristics of venules in response to important inflammatory stimuli.

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References


