Interactions between the sorbitol pathway, non-enzymatic glycation, and diabetic vascular dysfunction

Yasuo Ido1, Charles Kilo2 and Joseph R. Williamson1

Departments of 1Pathology and 2Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA

Abstract. Background. Many lines of evidence attest to a multifactorial pathogenesis of diabetic complications in humans and in animal models of diabetes. Increased sorbitol pathway metabolism and non-enzymatic glycation products have been implicated by many investigators in the pathogenesis of vascular and neural dysfunction as well as early vascular structural changes in animal models of diabetes. The present studies were undertaken to assess the mechanisms that mediate vascular dysfunction associated with these biochemical imbalances.

Methods. Three different animal models of diabetes were used: (1) rats with diabetes induced by injection of streptozotocin; (2) non-diabetic rats with acute hyperglycaemia of 5 h duration induced by i.v. glucose infusion at a rate sufficient to produce plasma glucose levels comparable to those in diabetic rats; and (3) the skin chamber granulation tissue model in which vessels in the chamber are exposed to buffer containing 5 or 30 mM glucose ± pharmacological agents or 0.1 mM glycated rat serum albumin ± pharmacological agents. Vascular function was assessed by injection of 11.3 μm 46Sc microspheres for quantification of blood flow and by injection of [125I] and [131I]bovine serum albumin for quantification of vascular albumin permeation.

Results. Vascular dysfunction induced by elevated glucose levels (increased blood flow and increased albumin permeation) in all three models was prevented by inhibitors of sorbitol pathway metabolism, inhibitors of nitric oxide synthesis and inhibitors of prostaglandin synthesis. In the skin chamber model vascular dysfunction induced by elevated glucose levels and by glycated rat serum albumin was prevented by superoxide dismutase, probucol and inhibitors of nitric oxide synthase.

Conclusions. These observations suggest that vascular dysfunction induced by increased sorbitol pathway metabolism (caused by elevated glucose levels) and by products of non-enzymatic glycation (at normal glucose levels) is mediated by a common final pathway consistent with a scenario in which: ↑ superoxide production → ↑ intracellular calcium levels → ↑ nitric oxide synthesis → ↑ blood flow and ↑ vascular permeability.

Key words: blood flow; diabetic microangiopathy; nonenzymatic glycation; oxidative stress; reductive stress; sorbitol pathway

Introduction

Many investigations have provided evidence linking early vascular and neural dysfunction and structural abnormalities in animal models of diabetes to increased sorbitol pathway metabolism, non-enzymatic glycation, reductive stress and oxidative stress [1–5]. The studies summarized here address the possibility that the effects of these different metabolic imbalances on vascular and neural dysfunction may be mediated by a common pathway. We have utilized three different animal models of diabetes in these studies: (1) rats with streptozotocin-induced diabetes in which the vasculature and nerves are exposed to a milieu like that in poorly controlled insulin-dependent diabetes; (2) rats with acute hyperglycaemia of ~5 h duration in which the tissues are exposed to hyperglycaemia (comparable to that in diabetic rats, i.e. plasma glucose levels of ~25 mM) and elevated insulin levels; and (3) the skin chamber granulation tissue model in which a circle of skin is removed from the back of non-diabetic rats and the vessels that form inside the chamber are exposed twice daily to elevated glucose levels, rat serum albumin glycated in vitro or other interventions at normal glucose levels. In this latter model the vasculature can be exposed to elevated glucose levels in the absence of metabolic and hormonal imbalances associated with
the diabetic milieu or with systemic hyperglycaemia in non-diabetic rats.

Methods

The effects of elevated glucose levels, sorbitol and glycated rat serum albumin on blood flow and albumin permeation were assessed in the three different animal models of diabetes described in Introduction. Since the methods for preparing these models have been described in detail except for the induction of acute hyperglycaemia, they will be described very briefly as follows.

Diabetes was induced by injection of 55–60 mg of streptozotocin in the femoral vein of male Sprague–Dawley rats anaesthetized with metophane. Acute hyperglycaemia was induced in conscious normal male Sprague–Dawley rats (250–300 g body wt) by infusion of 25% glucose (22.2 mmol/kg body wt/h) in water via a catheter in the vena cava which was inserted the day before the experiment under anaesthesia induced by i.p. injection of a mixture of 26 mg ketamine, 5.1 mg xylazine hydrochloride and 0.9 mg acepromazine/kg body wt. The rats recover within ~20 min and are allowed food and water prior to glucose infusion on the following morning. Control rats are infused with normal saline at the same volumetric rate as for glucose.

Tissue chambers are prepared by removal of an ~2 cm diameter circle of skin from either side of the lower back/thigh and a plastic chamber is sutured to the surrounding cuff of skin [6]. One week later (after new granulation tissue vessels form from fascia exposed inside the chamber) solutions containing 5 or 30 mM glucose ± pharmacological agents are added to the chambers twice daily (in a volume of 1–1.5 ml of HEPES buffer, pH 7.4) for 6–7 days, at which time blood flow and vascular albumin permeation are assessed in the granulation tissue vessels by injection of radiolabeled albumins and microspheres [4].

Regional blood flows were assessed by the reference sample microsphere method by injection of 11.3 μm ⁶⁶Sc microspheres [4]. Vascular permeation by [¹²⁵I]bovine serum albumin was quantified by a double isotope method by injection of [¹²⁵I]albumin (circulation time 10 min) and [¹³¹I]albumin (circulation time 2 min to correct for intravascular content of [¹²⁵I]albumin) [4]. Rats were anaesthetized with thiopental (80 mg/kg body wt) injected i.p. for these assessments of vascular function.

Rat serum albumin was obtained from Sigma (St Louis, MO). The albumin was incubated at a concentration of 50 mg/ml in 50 mM glucose in phosphate buffered saline (150 mM phosphate) containing 0.5 mM EDTA at 37°C for 4 weeks and was then dialysed against saline prior to addition to the chambers. Fluorescence at 370/440 nm was 257 for the glycated albumin versus 83 for albumin incubated under identical conditions in the absence of glucose.

Results

Inhibitors of aldose reductase and sorbitol dehydrogenase prevent vascular dysfunction induced by elevated glucose levels in all three models of diabetes used in these studies [3,4]. In addition they prevent impaired motor nerve conduction velocity in diabetic rats [3,4,8]. These inhibitors also prevent reductive stress, i.e. an increased ratio of cytosolic free NADH/NAD⁺, associated with increased flux of glucose via the sorbitol pathway [3–5].

**Fig. 1.** Putative roles of diabetes-induced reductive stress and non-enzymatic glycation in superoxide production and mechanisms that mediate vascular dysfunction induced by superoxide. Several lines of evidence (see text) are consistent with a scenario in which increased amounts of superoxide are produced by non-enzymatic glycation products as well as from metabolic imbalances associated with the diabetic milieu, i.e. increased oxidation of sorbitol to fructose. Extracellular superoxide is transported into cells through anion channels and increases intracellular calcium and prostaglandin synthetic activity. The increased intracellular calcium also will activate the constitutive isoform of nitric oxide synthase, thereby increasing blood flow and vascular permeability.
In diabetic rats vascular dysfunction (both increased blood flow and increased albumin leakage) is also prevented by inhibitors of prostaglandin synthesis [9] and by aminoguanidine, which inhibits formation of advanced glycation products as well as nitric oxide synthase [10-12].

In acutely hyperglycaemic rats increased blood flow in retina and sciatic nerve is prevented by inhibitors of prostaglandin synthesis, inhibitors of nitric oxide synthesis (N\textsuperscript{G}-methyl-l-arginine and aminoguanidine) and co-infusion of pyruvate, as well as by inhibitors of aldose reductase and sorbitol dehydrogenase [7,13; unpublished observations].

In the skin chamber granulation tissue model vascular dysfunction (increased blood flow and increased albumin leakage) induced by exposure to 30 mM glucose is also prevented by the same interventions that prevent hyperglycaemia-induced increases in blood flow in retina and nerve [3,6,10,14; unpublished observations]. In addition, 30 mM glucose-induced vascular dysfunction is prevented by co-administration of superoxide dismutase (150 U/ml buffer) and by administration of 3% probucol in the diet [14,15; unpublished observations]. Addition of 1 mM sorbitol (at normal glucose levels) to the chamber induces vascular dysfunction similar to that induced by 30 mM glucose; this vascular dysfunction is prevented by inhibition of sorbitol dehydrogenase (but not aldose reductase) and by all of the other interventions that prevent vascular dysfunction induced by 30 mM glucose [16; unpublished observations].

Addition of 0.1 \textmu M glycated rat serum albumin (in buffer containing 5 mM glucose) causes increased blood flow and increased albumin leakage similar to that induced by elevated glucose levels, whereas the non-glycated control albumin has no effect. These effects of glycated rat serum albumin are prevented by superoxide dismutase and the free radical scavenger probucol as well as by inhibitors of nitric oxide synthase [15; unpublished observations].

Addition of a superoxide generating system (xanthine oxidase + hypoxanthine) to the chamber at normal glucose levels also causes increased blood flow and increased vascular leakage similar to that caused by elevated glucose levels and by glycated albumin. These vascular changes are prevented by co-administration of inhibitors of nitric oxide synthase (aminoguanidine, N\textsuperscript{-}nitroarginine) as well as by superoxide dismutase [unpublished observations].

Addition of NO releasing agents to the chamber also increases blood flow and vascular permeability; these effects are unaffected by superoxide dismutase [unpublished observations].

Discussion

These observations indicate that blood flow and vascular leakage are increased by: (1) metabolic imbalances caused by elevated glucose levels per se independent of metabolic and hormonal imbalances associated with the diabetic milieu and with systemic hyperglycaemia in non-diabetic rats; and (2) products of non-enzymatic glycation.

Observations in the tissue chamber model support the likelihood that increased production of superoxide and nitric oxide play important roles in mediating these manifestations of vascular dysfunction. The findings in the tissue chamber model are consistent with the scenario depicted in Figure 1 by which increased superoxide levels may mediate increased blood flow and vascular leakage. Increased superoxide levels have been reported to increase intracellular calcium levels and activate prostaglandin synthesis in cultured human amniotic cells [3,5,17,18]. Increased intracellular calcium levels also will activate the constitutive isoform of nitric oxide synthase, resulting in increased blood flow. Although glycated proteins have been reported to scavenge nitric oxide in vitro [19], there is no evidence that this reaction is quantitatively significant in vivo. In the publication by Bucala et al. [19] the beneficial effects of aminoguanidine on blood pressure responses to acetylcholine may be due to increased vascular compliance as a result of decreased collagen cross-linking, as reported by Brownlee et al. [20]. Other reports of decreased nitric oxide production in diabetic rats based on the use of nitric oxide synthase inhibitors are flawed because non-selective inhibitors of NO synthase were used and the animals became hypertensive [21,22]. It is well known that inhibition of constitutive endothelial nitric oxide synthase is associated with procoagulant changes and adhesion of leukocytes in addition to causing hypertension [23].

Many metabolic imbalances associated with the diabetic milieu may cause reductive stress. The best characterized example is increased metabolism of glucose via the sorbitol pathway [3-5]. In the second step of this pathway oxidation of sorbitol to fructose is coupled to reduction of NAD\textsuperscript{+} to NADH by sorbitol dehydrogenase, resulting in a hypoxia-like increase in cytosolic free NADH/NAD\textsuperscript{+}, i.e. reductive stress. In hypoxic tissues this same reductive stress results from impaired oxidation of NADH to NAD\textsuperscript{+}. It is noteworthy that regional hypoxia, like increased blood elevated glucose levels and glycated albumin, also increases blood flow.

Increased superoxide production by products of non-enzymatic glycation has been reported by numerous investigators [1]. Reductive stress also favours increased superoxide production by several mechanisms [3,5,24-26], as shown in Figure 1. Superoxide is a natural by-product of prostaglandin synthesis which is increased in animal models of diabetes [3]. Since NADH is a potent inhibitor of xanthine dehydrogenase, an increased ratio of NADH/NAD\textsuperscript{+} will favour metabolism of purine nucleotides via xanthine oxidase with associated increased production of superoxide [26]. NAD(P)H oxidases also produce superoxide [3]. NADH has been reported to release Fe\textsuperscript{2+} from ferritin, which would favour increased production of superoxide via the Fenton reaction [25]. Lastly, NADH is much more susceptible than NAD\textsuperscript{+} to autoxidation with
release of superoxide [3]. Further studies are needed to determine the quantitative importance of these different mechanisms to increased superoxide production under these experimental conditions as well as in human diabetics.

The putative important role for increased superoxide levels in mediating vascular dysfunction in these experiments is consistent with evidence of oxidative stress in diabetic humans and animals, and with evidence that free radical scavengers and antioxidants appear to ameliorate functional abnormalities in animal models of diabetes and decrease the levels of products of oxidative damage [1,3].

The finding that vascular dysfunction induced by elevated glucose and sorbitol levels is prevented by inhibitors of sorbitol pathway metabolism as well as by superoxide dismutase and inhibitors of nitric oxide synthase is consistent with the likelihood that increased flux of glucose via the sorbitol pathway increases superoxide production as a consequence of hypoxia-like reductive stress resulting from reduction of NAD$^+$ to NADH coupled to oxidation of sorbitol to fructose.

Acknowledgements. This research was supported by National Institutes of Health Grants EY-06600, HL-39934 and DK-20579, and by the Kilo Diabetes and Vascular Research Foundation.

References

7. Hasan KS, Santiago JV, Williamson JR. Acute hyperglycaemia-induced increases in regional blood flow are prevented by Pyruvate and by Tolrestat. (ADA abstract.) Diabetes 1993; 42(Suppl I): 189A
15. Tilton RG, Chang K, Faller A. Probucol prevents vascular protein leakage induced by diabetes, glucose, and glycated proteins (ADA abstract.) Diabetes 1993; 42(Suppl I): 89A