Simulating the Diabetic Environment Modifies In Vitro Prostacyclin Synthesis

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SUMMARY

To explain the contradictory data on the secretion of prostacyclin (PGI_2) in clinical and experimental diabetes, we have investigated the effect of each of the major metabolic abnormalities in uncontrolled diabetes on vascular PGI_2 synthesis. An increase in fatty acid concentrations caused a dose-dependent inhibition of PGI_2 synthesis by rat aortic rings in vitro; linoleic and linolenic acids were consistently the most and palmitic the least inhibitory. Glucose had a stimulatory effect between 10 and 30 mmol/L, but a progressive fall in stimulation occurred at higher concentrations. Insulin was inhibitory at 10 and 50 mU/L; however, in combination with glucose (10 mmol/L) it was significantly stimulatory (at 10 mU/L) when the aortic rings were preincubated for 2 and 4 h. A pH of 7.0 or less was significantly inhibitory, whereas ketone bodies had no significant effect on PGI_2 synthesis. These data show for the first time that altered metabolic factors in uncontrolled diabetes may have different effects on vascular PGI_2 synthesis. These data help explain the variability of observations related to PGI_2 synthesis and secretion in diabetes, and advocate a more detailed definition of the metabolic status of patients/animals used in such experiments. DIABETES 32:217–221, March 1983.

The association of diabetes mellitus with platelet hyperaggregability,[1–3] on the one hand, and vascular disease[4] on the other, has led to considerable investigation of the role of the former in the pathogenesis of the latter. Among the possible factors influencing platelet hyperaggregability is impaired secretion of prostacyclin (PGI_2) by the vascular endothelium. PGI_2 is a vasodilator and is the most potent naturally occurring inhibitor of platelet aggregation.[5] It has been suggested that changes to the vascular endothelium of diabetic patients result in diminished production of PGI_2, contributing to platelet hyperaggregability. Evidence to support this hypothesis is equivocal, since plasma concentrations of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2) have been shown to be elevated,[6] diminished[7] or normal,[8] in diabetic patients. Data obtained from the blood vessels of animals rendered diabetic are also conflicting.[9–11]

Since the state of diabetes consists of several metabolic abnormalities which are in constant flux, we considered the possibility that the previous conflicting reports may relate to this changing internal environment. We therefore investigated the effect on in vitro PGI_2 production of each of the major metabolic factors known to be abnormal in diabetes.

MATERIALS AND METHODS

Minimum Essential Medium (MEM), with Hanks Salts and L-Glutamine, was purchased from Gibco Bio-Cult Ltd. (Grand Island, New York). Oleic, palmitic, linoleic, linolenic and stearic acids and fatty acid free human albumin were obtained from Sigma Chemical Co. (St. Louis, Missouri), 1-[^{14}C]-arachidonic acid (specific activity 58.4 mCi/mmol; ^{14}C-AA) from New England Nuclear (Boston, Massachusetts), and Actrapid insulin from Novo Industries (Copenhagen, Denmark). All other reagents were purchased from BDH Chemical Co. Ltd., Poole, England. Kit for measuring nonesterified fatty acids was obtained from Boehringer Mannheim.

METHODS

Male Sprague Dawley rats (200–250 g) were decapitated. Their abdomens were opened by a midline incision, and their aortae were rapidly removed, rinsed in chilled Krebs Ringer bicarbonate solution (gassed with 95% O_2, 5% CO_2 to pH 7.4), and freed of fatty tissue and adventitia. The aortae were cut into 1-mm rings and incubated in two phases: a *test incubation phase* lasting up to 6 h in MEM containing those components that were being tested and would mimic the "diabetic" microenvironment; and an *arachidonate incuba-*

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tion phase lasting 90 min with ^{14}C-AA dissolved in Tris buffer to evaluate PGI_2 production.

Test incubation phase. The aortic rings were placed in stoppered tubes (100 mg per tube) containing 10 ml MEM, pH 7.4 (pregassed with 95% O_2, 5% CO_2, and containing $NaHCO_3$ 25 mmol/L). The tubes were incubated for up to 6 h with the following additions or changes to the medium: (1) 5–100 mmol/L glucose or 5–100 mmol/L sucrose; (2) 1–100 mU/L Actrapid insulin; (3) glucose and insulin at optimal concentrations obtained from (1) and (2); (4) palmitic, stearic, linoleic, oleic, or linolenic acids were added as set out below; (5) sodium acetoacetate, acetone, or sodium-3-hydroxybutyrate from 30 mg/L to 3 g/L; (6) altered pH by addition of HCl or NaOH over a range of 6.8–8.0; (7) potassium (as KCl) from 2 to 20 mmol/L.

Fatty acids were dissolved in warm ethanol. Five microliters of these solutions were added to 1 ml of 10% (wt/vol) fatty acid free human albumin solutions in 0.1 mM phosphate buffer at pH 7.4. The mixtures were shaken thoroughly: no turbidity or separation into two phases was observed. Measurement of free fatty acid concentrations in these solutions revealed between 75 and 93% of the fatty acids added, as measured with a kit from Boehringer Mannheim.

The pH of the media, except in experiment 6, was maintained at 7.4.

The test incubation phase was always followed by an arachidonate incubation phase.

Arachidonate incubation phase. Following the test incubation phase, the aortic rings were rinsed thoroughly in Tris buffer, pH 8.0. Fifteen milligrams of aortic rings were placed in sextuplicate polyethylene tubes, and 5 nCi of ^{14}C-AA (=856 nmol/L of AA) in 100 μl Tris buffer, pH 8.0, was added to each tube. The tubes were incubated at 37°C in a water bath for 90 min. The supernatants were removed, and 200 μl of ethanol was added; the contents were immediately placed in a freezer at −70°C. At a later date, unchanged ^{14}C-AA was separated from 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2) by thin layer chromatography, and percentage (%) conversion calculated.[12] In additional experiments, the test compounds were added directly into the arachidonate incubation phase, without a prior test incubation phase.

EXPRESSION OF RESULTS

The percent conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$ was determined in control (C) and test "diabetic environment" (D) experiments. The results are expressed as the percent increase or decrease in conversion, which was determined using the formula:

$$\frac{D - C}{C} \times 100.$$

STATISTICAL ANALYSIS

Paired *t* tests were used to derive P values. The percent conversion obtained in control experiments was compared with the percent conversion obtained in test experiments. C experiments consisted of test and arachidonate incubation phases with no alteration to the basic contents of the incubates. D experiments consisted of test and arachidonate incubation phases with the compounds being tested added to the basic contents of the appropriate incubates. The P values are shown in the text or with the appropriate figures or tables.

RESULTS

The basal ^{14}C-AA incorporation into 6-keto-$PGF_{1\alpha}$ by control aortic rings was consistently 15 ± 2% in our 50 separate experiments, each with six observations.

Experiment 1—incubation with glucose alone (Figure 1). Following 6-h test incubation in MEM, a dose-response curve was obtained. Maximal conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$ was seen at glucose concentrations between 10 and 30 mmol/L decreasing at 50 mmol/L of glucose to conversion not significantly different from controls. No significant effect was seen when glucose was added directly during the arachidonate incubation phase with ^{14}C-AA over a concentration range of 1 mmol/L to 100 mmol/L of glucose.

The effect of osmolality was examined by preincubating the aortic rings for 6 h in medium containing the same concentrations of sucrose as those used for glucose. Sucrose proved to have no effect on the conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$, up to 100 mmol/L of sucrose.

Experiment 2—incubation with insulin alone (Figure 2). Direct addition of insulin to the arachidonate incubation phase had a significant inhibitory effect from 10 mU/L ($P < 0.01$) to 50 mU/L ($P < 0.0002$). The effect of insulin added to the test incubation phase was also examined in the presence of glucose, as described in experiment 3.

Experiment 3—incubation with insulin and glucose (Table 1). Using 10 mmol/L of glucose with varying doses of insulin added to the test incubation phase, no significant differences were seen after 1-h preincubation, whereas at 2 h, maximal conversion was observed at insulin concentrations of 10 mU/L. At 50 mU/L of insulin, this stimulatory effect disappeared, and the conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$ was not significantly different from controls. Following 4-h test incubation, conversion at all insulin concentrations was equally elevated above controls, except at 50 mU/L, when

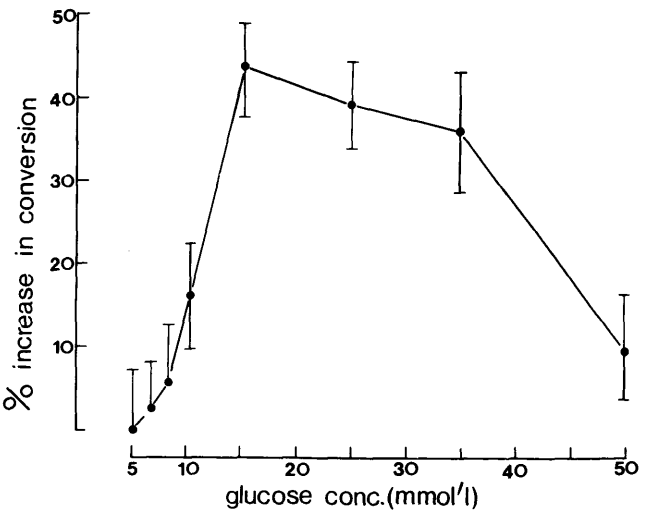

FIGURE 1. Effect of glucose on conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$ after 6-h test incubation. Significant ($P < 0.001$) increase in conversion only occurred at glucose concentrations of 15, 25, and 35 mmol/L. The data are presented as percent change over basal controls.

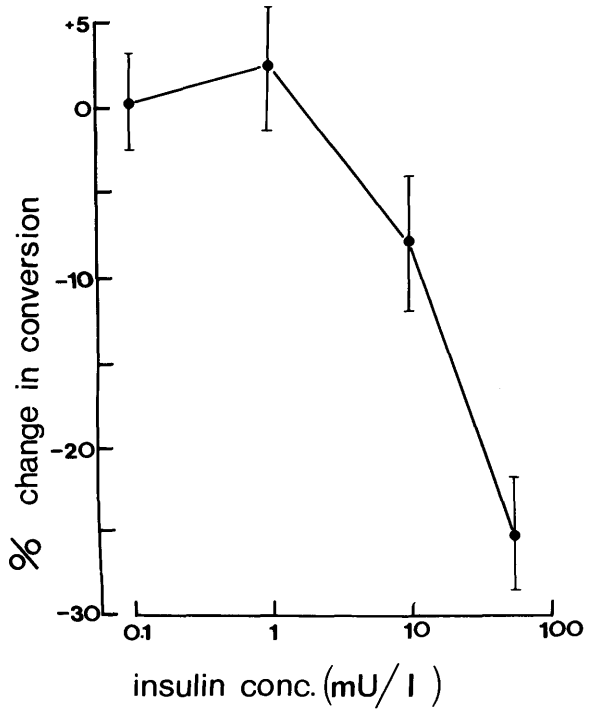

FIGURE 2. Effect of insulin concentration on conversion of ^{14}C-AA to 6-keto-PGF$_{1\alpha}$ after addition of insulin to arachidonate incubation phase. The data are presented as percent change over basal controls.

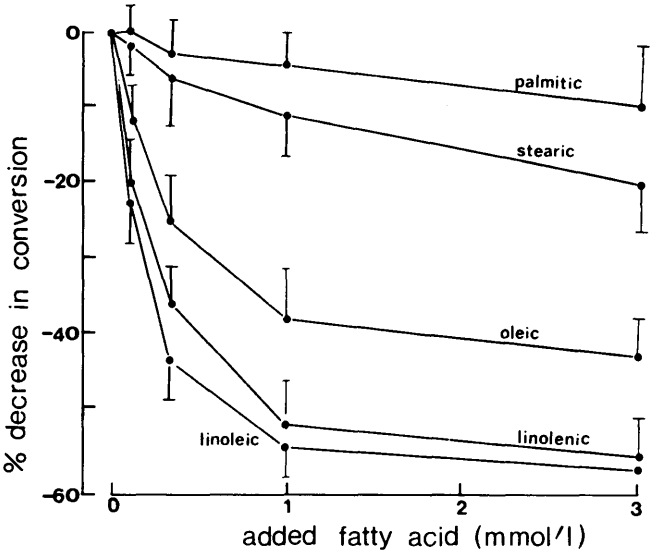

FIGURE 3. Effect of fatty acids on conversion of ^{14}C-AA to 6-keto-PGF$_{1\alpha}$ after 6-h test incubation. The data are presented as percent change over basal controls. Recoverable fatty acid varied between 75 and 93% of the amount added.

conversion was below optimal but not significantly different from controls.

Experiment 4—incubation with fatty acids (Figure 3). Fatty acids over a concentration range of 100 μmol/L to 3 mmol/L were all inhibitory in a dose-dependent fashion after 6-h test incubation. The order of potency of the fatty acids was linoleic = linolenic > oleic > stearic > palmitic. Palmitic acid did not achieve significant inhibition of ^{14}C-AA conversion to 6-keto-PGF$_{1\alpha}$, whereas the other saturated fatty acid, stearic acid, was significantly ($P = 0.001$) inhibitory only at the highest concentration tested (3 mmol/L). Unsaturated fatty acids achieved significant inhibition at 100 μmol/L ($P < 0.001$, linolenic and linoleic) and at 300 μmol/L ($P < 0.001$, oleic acid).

Similar inhibition was observed when the fatty acids were included in the arachidonate incubation phase.

Experiment 5—incubation with ketone bodies. Acetone, sodium acetoacetate and 3-hydroxybutyrate had no effect on ^{14}C-AA metabolism at concentrations of up to 3 g/L when added to the incubation phase (6 h). Similarly, no effect was observed when these ketone bodies were included in the arachidonate incubation phase.

Experiment 6—incubation at various pHs (Table 2). Significant decreases in the incorporation of ^{14}C-AA into 6-keto-PGF$_{1\alpha}$ were seen following 6-h test incubation phase at pH 7 or below. Maximal incorporation of ^{14}C-AA was observed at pH 7.7.

Optimal pH has been established for the arachidonate incubation phase at 8.0.[12]

Experiment 7—incubation with potassium (Figure 4). Direct addition of potassium to the arachidonate incubation phase had a significant stimulatory effect at concentrations of 2 ($P = 0.04$), 4 ($P < 0.001$) and 8 mmol/L ($P = 0.002$). Since MEM contains 5.6 mmol/L of potassium, the effect of potassium over 6-h preincubation was examined at final concentrations of potassium of 10, 15, and 20 mmol/L. Significant stimulation above controls was seen at 10 ($P < 0.002$)

TABLE 1
Effect of varying insulin dose at a constant glucose concentration (10 mmol/L) on percent conversion (±SD) of ^{14}C-AA into 6-keto-PGF$_{1\alpha}$ for varying durations

Duration of test incubation	Insulin concentration (mU/L)				
	0	1	5	10	50
1 h	3.4 ± 0.7 (NS)	12.6 ± 2.0 (NS)	5.8 ± 0.7 (NS)	10.8 ± 1.7 (NS)	12.6 ± 1.4 (NS)
2 h	8.6 ± 1.8 (NS)	9.1 ± 1.8 (NS)	18.7 ± 2.6 (0.005)	28.4 ± 5.6 (0.002)	7.9 ± 0.3 (NS)
4 h	18.6 ± 2.9 (0.005)	24.6 ± 3.2 (0.002)	20.8 ± 2.8 (0.002)	23.6 ± 2.5 (0.002)	9.5 ± 0.8 (NS)
6 h	22.2 ± 3.1	—	—	—	—

Figures in parentheses represent P values. The data are presented as percent change over the conversion by basal controls. NS = not significant.

TABLE 2
Effect of pH on percent changes in conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$ (±SD) following 6-h test incubation

pH	% change in conversion	P
6.8	−28 ± 4.6	<0.001
7.0	−18.0 ± 1.6	<0.002
7.2	−1.7 ± 0.35	NS
7.4	Zero control	—
7.7	+8.6 ± 1.3	NS

The data are presented as percent change over the basal controls. NS = not significant.

and 15 mmol/L ($P < 0.002$) of potassium, whereas conversion was significantly reduced ($P < 0.001$) at 20 mmol/L of potassium.

DISCUSSION

Although others have demonstrated that experimental diabetes in the rat is associated with altered PGI_2 production,[9-11] the present study analyzed separately in vitro, the role of each of the major abnormal biochemical factors occurring in uncontrolled diabetes.

Our results show that at least two of the variables investigated were potent inhibitors of the incorporation of arachidonate into 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2): elevated fatty acid concentrations and low pH. In addition, high insulin concentrations, which may be transiently associated with episodes of hypoglycemia in poorly controlled diabetes, also caused an inhibition of PGI_2 synthesis. In contrast, high concentrations of ketone bodies (3-hydroxybutyrate, acetoacetate, and acetone) did not have a significant effect on PGI_2 synthesis. On the other hand, what would be clinically unacceptable plasma glucose concentrations (10–30 mmol/L) significantly stimulated PGI_2 synthesis, while 6-keto-$PGF_{1\alpha}$ production at higher concentrations of glucose (50 mmol/L) did not differ significantly from that in controls. Very high glucose concentrations are known to occur in nonketotic hyperosmolar crises,[13] a condition known to be associated with an increased incidence of thrombosis.[14] This effect of glucose was not mediated by increased osmolality, since sucrose was ineffective. Potassium was stimulatory at both physiological concentrations and those expected in diabetic ketoacidosis.

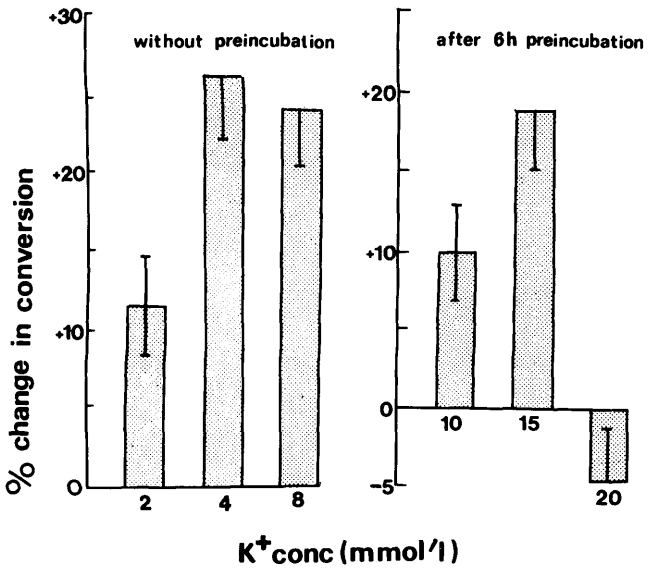

FIGURE 4. Effect of potassium concentration on conversion of ^{14}C-AA to 6-keto-$PGF_{1\alpha}$ during (a) arachidonate incubation phase (90 min) and (b) test incubation phase (6 h). The data are presented as percent change over basal controls.

The inhibitory effect of fatty acids on PGI_2 synthesis was the most impressive of the various factors investigated, since it was apparent at concentrations modestly higher than normal that occur in diabetics only marginally out of control.[15] Although all fatty acids tested produced a significant inhibition of PGI_2 synthesis, unsaturated fatty acids (oleic, linoleic, and linolenic) were more effective. This is probably related to the greater similarity in structure of unsaturated fatty acids to arachidonic acid, a polyunsaturated fatty acid. We have previously demonstrated that fatty acids, especially the unsaturated ones, enhance the rate of degradation of PGI_2 in plasma and in albumin solutions.[16] Increased fatty acid concentration thus has a dual effect, influencing both PGI_2 synthesis and degradation. This may result in diminished PGI_2 concentration in plasma, which may in turn contribute to enhanced platelet aggregability.

Low pH, 7.0 and below, was also associated with significantly diminished PGI_2 synthesis. This is of relevance, since such pH may occur in diabetic ketoacidosis, a condition also known to be associated with an increased incidence of thrombosis.[14] Interestingly, ketone bodies did not exert a detrimental effect on this system, independently of pH.

Insulin-induced hypoglycemia in normal subjects has been shown by us to result in enhanced platelet aggregation.[17] These findings were confirmed by others in diabetic patients.[18] It is therefore of interest that we observed that high concentrations of insulin inhibited PGI_2 synthesis by the rat aortic rings. Similar PGI_2 synthesis and platelet aggregation changes may occur during hypoglycemic episodes in diabetics, when excessive insulin is present in the circulation. The recent report that hypoglycemic episodes in diabetics were associated with transient ischemic attacks[19] lends some support to the concept that platelet hyperaggregability may play a role in the pathogenesis of transient ischemic episodes associated with hypoglycemia. Furthermore, hyperinsulinism is also associated with atherosclerosis,[20] a process where platelet adhesion may play an important role. Further work is obviously required to validate the clinical significance of our in vitro observations.

Another point raised by our observations is relevant to the contradictory findings of other workers in relation to PGI_2 production by blood vessels from diabetics and the plasma concentrations of 6-keto-$PGF_{1\alpha}$ in these patients. These contradictory observations could well be the consequence of the constantly changing homeostatic factors regulating PGI_2 secretion as demonstrated by our experiments.

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