Effects of Epicatechin on Rat Islets of Langerhans

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SUMMARY

Plants containing epicatechin (a flavonoid) have been used to treat diabetes mellitus in Indian medicine. The present study reports effects of this compound on isolated islets of Langerhans. The flavonoid (1 mM) was found to increase insulin secretion from isolated rat islets of Langerhans in the presence of either 2 or 20 mM glucose, in static incubations, or in perifusion. The increase in insulin secretion mediated by epicatechin was both ATP- and temperature-dependent. Ultrastructural studies showed no deleterious changes in the structure of the B-cells after 5 days of exposure to the compound. Intraperitoneal injection of 30 mg/kg body wt of epicatechin twice daily for 4 days increased the islet insulin content by 30%. Secretion of insulin from islets isolated from epicatechin-injected rats was significantly increased when exposed to 20 mM glucose in comparison with water-injected controls. Furthermore, islets of adult rats cultured with 5.5 mM glucose for 4 days showed a significant increase in DNA synthesis in the presence of 0.05 mM epicatechin. These results suggest direct effects of epicatechin on various aspects of islet function. DIABETES *33*:291–296, March 1984.

Herbs and plant extracts have traditionally been used as cures for diabetes mellitus in folk medicine all over the world. It was reported recently that the bark of *Pterocarpus marsupium Roxb.* had been used successfully in Indian medicine for the treatment of diabetes.[1] The active antidiabetic agent of the plant, suggested to be (−)epicatechin,[2,3] a benzopyran, protected normal rat islets from alloxan, brought about a normalization of blood glucose levels, and promoted B-cell regeneration in islets of alloxan-treated rats.[1] This article describes an investigation of the effects of (−)epicatechin on insulin secretion from isolated islets of Langerhans, on total islet insulin and protein content after injection of rats with the agent, and on secretion and DNA synthesis in adult rat islets that were cultured with epicatechin.

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MATERIALS AND METHODS

MEDIA

Islets were isolated in an ice-cold, bicarbonate-buffered medium,[4] supplemented with 2 mM glucose and equilibrated to pH 7.4 with 95% O_2 and 5% CO_2. For culture purposes, sterile Hanks' buffered salt solution (HBSS) (Gibco, Grand Island, New York), supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml) (Glaxo, Greenford, United Kingdom), and sodium bicarbonate (2.2 g/L) was used for isolation and subsequent secretion experiments. Culture of islets was performed in Medium 199 (Gibco) with modified Earle's salts, L-glutamine, and phenol red. Media contained 5.5 mM glucose or 20 mM glucose, were buffered with 25 mM sodium bicarbonate and supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml), plus 10% heat-inactivated, virus- and mycoplasma-screened fetal calf serum (Gibco). In addition, test culture media contained 0.05 mM (−)epicatechin (Sigma Chemical Co., Poole, Dorset, United Kingdom), dissolved in absolute ethanol and/or 12.5 mM hydroxyurea. A similar volume of the solvent above was added to control cultures.

ISOLATION OF ISLETS

Islets were isolated from male WAG rats weighing 150–250 g by collagenase digestion.[5] Collagenase was purchased from Sigma Chemical Co. and Boehringer (Lewes, Sussex, United Kingdom). For in vivo experiments, animals weighing 100–150 g were used.

INSULIN SECRETION

Static secretion. After isolation, the islets were preincubated in medium containing 2 mM glucose for 30 min at 37°C. At

the end of this period, they were distributed into medium containing 1 mg/ml albumin (Armour Pharmaceuticals Ltd., Eastbourne, Sussex) and the following additions: 2 mM glucose with or without 1 mM (−)epicatechin dissolved in absolute ethanol, 0.5 mM ascorbic acid and 0.25 mM 2,4-DNP, or 20 mM glucose with or without the same supplements. After 1-h incubation at 37°C or 4°C in a shaking water bath, samples were withdrawn for insulin determination.

Perifusion. Islets were preincubated for 30 min at 37°C in medium containing 2 mM glucose. Groups of 50 islets were then placed on a 10-μm mesh nylon filter (Plastock Ltd, Birkhead) in a Swinnex filter holder (Millipore Corp.) and perifused by using a Gilson HP8 peristaltic pump (Anachem Ltd., Luton, Beds) with medium containing 2 mM glucose. Perifusion started with a flow rate of 4.8 ml/min for 5 min at 4°C to wash out insulin transferred with the islets as well as insulin secreted before the start of perifusion. The flow rate was then adjusted to 1 ml/min and the temperature raised to 37°C. Fractions of 2 ml were collected. An initial 5 fractions were collected to allow a basal secretion to be established. Perifusion was continued with media containing 2 mM or 20 mM glucose, with or without epicatechin. All fractions collected at 37°C were later assayed for insulin content.

Insulin immunoassay. The insulin content of media was determined by radioimmunoassay, using guinea pig anti-insulin serum kindly donated by Dr. W. Montague, University of Leicester, rat insulin standards purchased from Novo Laboratory, Denmark, and ^{125}I-labeled bovine insulin iodinated in our laboratory by a chloramine-T method.[6] Antibody-bound radioactivity was separated from unbound by precipitation in 12% polyethylene glycol 6000 (BDH Chemicals, Poole, Dorset, United Kingdom).[7]

INJECTION OF ANIMALS

(−)Epicatechin, dissolved in water, was injected in doses of 30 mg/kg intraperitoneally twice a day for 4 days into male WAG rats (100–150 g). The controls were injected with a similar amount of water. At the end of 4 days, the animals were killed and their islets isolated by the collagenase method.

TOTAL PROTEIN AND INSULIN ESTIMATION

Groups of 100 islets were transferred into 0.6 ml of 0.1 N NaOH and disrupted using the MSE Soniprep 150 ultrasonic disintegrator (MSE Ltd., Crawley, Sussex, United Kingdom). Samples were then withdrawn for protein determination.[8] For total insulin estimation, groups of 10 islets in acid ethanol were disrupted ultrasonically and samples withdrawn for insulin radioimmunoassay.

ISLET CULTURES

The pancreata were removed asceptically and placed in ice-cold, sterile HBSS and all subsequent work carried out in a clean air cabinet (Microflow Ltd., United Kingdom). After collagenase digestion, the digest was washed 3 times with HBSS. The washed digest was resuspended in HBSS and the islets picked using a sterile, drawn-out Pasteur pipette. Clean islets were then washed twice in HBSS and distributed into tissue culture flasks (Falcon) containing 50 ml of the appropriately supplemented Medium 199. The flasks were then incubated for 4 days at 37°C in a humidified atmosphere of 5% CO_2 (Leec MK 11; Leec Ltd., Nottingham, United Kingdom). The complete medium of each flask was changed after 2 days of culture. On the 4th day, the islets were detached from the bottom of the flasks by gently tapping the sides of the flasks. They were then harvested for static incubation experiments or for ^{3}H-thymidine labeling.

ESTIMATION OF DNA SYNTHESIS

After detachment from the flasks, the islets were washed once in fresh Medium 199. Groups of 120 islets were replated onto sterile culture dishes containing 2 ml of the appropriately supplemented Medium 199. After the addition of 5 μCi/ml of (methyl-^{3}H)-thymidine (specific activity of 6.7 Ci/mmol, New England Nuclear Corp.) to each group of islets, the dishes were cultured for a further 24 h at 37°C. Each group of islets was then washed twice in HBSS containing 10 mM unlabeled thymidine and disrupted ultrasonically in polythene Eppendorf tubes (Alpha Laboratories, Eastleigh, Hants, United Kingdom) containing 250 μl distilled water.

Separation of labeled DNA from unbound ^{3}H-thymidine was achieved by filtration through glass-fiber filters. Duplicate samples of homogenates were filtered through glass-fiber discs (GF/A 2.5 cm, Whatman, United Kingdom). The discs were then thoroughly dried and placed in scintillation vials containing 5 ml of scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole in toluene/Triton X-100 2:1, (v/v)]. The radioactivity attached to the discs was measured in a Beckman LS 7500 liquid scintillation spectrometer. Further duplicate samples of islet homogenates were taken for estimation of DNA content.[9–11] Incorporation of ^{3}H-thymidine was then expressed as cpm/μg islet DNA.

STATISTICS

Statistical significance was analyzed by Student's *t* test for paired or unpaired data as appropriate. All values are expressed as mean ± SEM of the number of observations indicated.

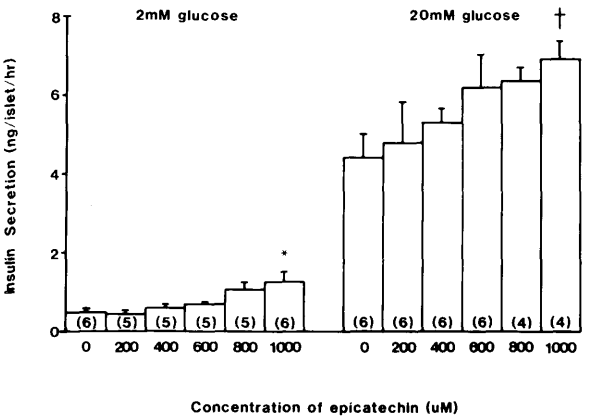

FIGURE 1. Effects of various doses of epicatechin on islets in static incubation. Various concentrations of epicatechin were tested on islets in the presence of 2 and 20 mM glucose. Experimental details are given in MATERIALS AND METHODS. Values are given as mean ± SEM of the numbers in parentheses, denoting the number of observations. Significance of difference between tests and appropriate controls: *P < 0.05, †P < 0.01.

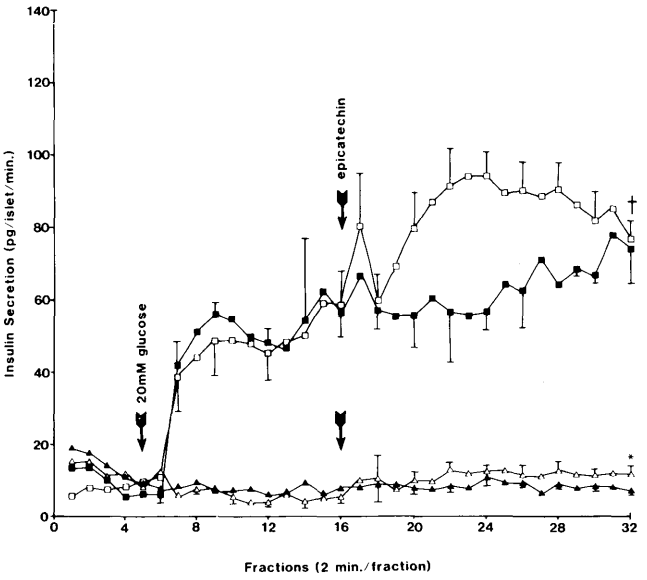

FIGURE 2. Effects of epicatechin (1 mM) on insulin secretion at 2 and 20 mM glucose from islets in perifusion. Islets were preincubated for 30 min before being transferred into perifusion chambers. Experimental details are given in MATERIALS AND METHODS. ▲, ■: Islets perifused with 2 and 20 mM glucose, respectively. △, □: Islets perifused with 2 and 20 mM glucose, respectively, and exposed to 1 mM epicatechin from the 16th fraction. Points on the curve represent the mean, and bars the SEM, of 7 perifusions. Integrated areas under the curves from the 16th to 32nd fractions were compared between tests and controls. Significance of difference: *P < 0.01, †P < 0.001.

RESULTS

(−)Epicatechin increased insulin secretion from isolated islets of Langerhans in static incubations (Figure 1) in a dose-dependent fashion. One millimolar epicatechin produced the greatest response from islets exposed to both low- and high-glucose medium. At this concentration of epicatechin, secretion was increased from 0.50 ± 0.08 to 1.22 ± 0.30 ng/islet/h ($P < 0.05$) at 2 mM glucose, and from 4.39 ± 0.59 to 6.89 ± 0.45 ng/islet/h at 20 mM glucose ($P < 0.01$, N = 4–6).

Similarly, perifusion experiments showed that 1 mM epicatechin significantly raised insulin secretion from islets responding to 2 mM and 20 mM glucose (Figure 2). Integrating the areas under the curves, from the 16th to 32nd fraction, inclusive, showed that insulin secretion rose from 8.54 ± 0.28 to 10.27 ± 0.45 pg/islet/min ($P < 0.01$, N = 7) at 2 mM glucose. Secretion in response to 20 mM glucose in the same period was increased from 63.18 ± 1.91 to 84.20 ± 2.35 pg/islet/min ($P < 0.001$, N = 7).

Epicatechin, being a flavonoid, was observed to undergo photo-oxidation after 1-h incubation, exhibiting a characteristic brownish yellow color. To prevent photo-oxidation and to determine if the oxidized form of epicatechin was responsible for the observed effects on secretion, 0.5 mM ascorbic acid was used,[12] as the latter is a reducing agent. A series of experiments was also performed to test if the epicatechin-mediated effects on secretion were energy- and temperature-dependent. Table 1 shows results of static incubations performed in the presence of 0.5 mM ascorbic acid, 0.25 mM 2,4-DNP at 37°C and 4°C.

At 4°C, the response to glucose was reduced by approximately 80% when compared with a similar response at 37°C ($P < 0.001$) (Table 1). At this temperature, epicatechin had no effect on secretion at 2 or 20 mM glucose. The presence of 0.5 mM ascorbic acid alone did not significantly alter the rate of secretion (Table 1), and photo-oxidation of epicatechin did not occur in the presence of ascorbic acid, as judged by the absence of a color change. However, no

TABLE 1
Effects of ascorbic acid, temperature, and 2,4-DNP on epicatechin-mediated insulin secretion

Temperature	Glucose conc. (mM)	Epicatechin (1 mM)	Ascorbic acid (0.5 mM)	2,4-DNP (0.25 mM)	Secretion (ng/islet/h)
4°C	2				0.56 ± 0.1 (3)
		+			0.57 ± 0.08 (3)
			+		0.57 ± 0.07 (3)
		+	+		0.69 ± 0.14 (3)
	20				0.91 ± 0.17 (3)†
		+			0.92 ± 0.17 (3)
			+		0.94 ± 0.17 (3)
		+	+		0.68 ± 0.17 (3)
37°C	2				0.61 ± 0.1 (4)
		+			1.55 ± 0.3 (4)*
			+		0.61 ± 0.2 (4)
		+	+		1.23 ± 0.22 (4)
		+		+	0.60 ± 0.11 (4)
	20				4.36 ± 0.75 (7)
		+			6.92 ± 0.86 (7)*
			+		4.11 ± 0.51 (4)
		+	+		6.60 ± 1.05 (5)
		+		+	1.42 ± 0.38 (5)†
				+	1.29 ± 0.22 (3)‡

Islets were incubated in the presence of 2 or 20 mM glucose, with or without 0.5 mM ascorbic acid, 0.1 mM epicatechin, and 0.25 mM 2,4-DNP at 4°C or 37°C. Experimental details are given in MATERIALS AND METHODS. Values are mean ± SEM. Significance of difference between controls and the various tests: *$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$. Numbers in parentheses are the number of observations.

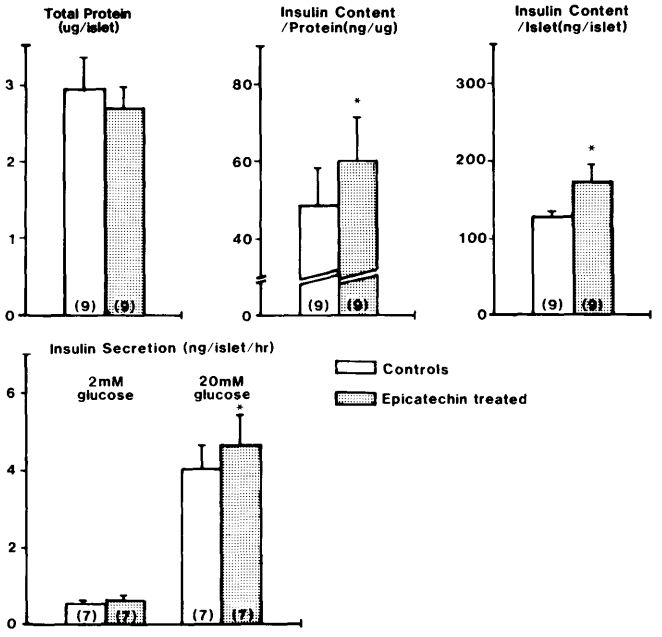

FIGURE 3. Effects of epicatechin injection on secretion, total protein, and insulin content. Rats were injected with epicatechin in doses of 30 mg/kg body wt twice daily for 4 days. They were then killed and their islets isolated for static incubations, total insulin, and protein determinations. Experimental details are given in MATERIALS AND METHODS. Values are mean ± SEM. Signficance of difference between control and islets from injected rats: *P < 0.05. Numbers in parentheses denote number of observations.

significant change was observed in the epicatechin-mediated secretion in the presence of ascorbic acid (Table 1).

ATP dependence of epicatechin-stimulated insulin secretion was demonstrated when 0.25 mM 2,4-DNP was added (Table 1). At 2 mM glucose, the effect of epicatechin on secretion was completely inhibited, and at 20 mM glucose secretion was reduced by approximately 80% ($P < 0.001$). Addition of 2,4-DNP significantly decreased glucose-stimulated secretion ($P < 0.01$).

The ultrastructure of B-cells from islets cultured for 5 days at 5.5 mM glucose plus 1 mM epicatechin appeared to be normal (results not shown). At this high concentration of epicatechin, the islets showed some signs of fragility after culture for > 24 h. This was greatly reduced by reducing the concentration of epicatechin to 0.05 mM in subsequent culture experiments.

The rate of insulin secretion from islets isolated from rats that had been injected with epicatechin twice daily for 4 days was higher than that from islets of control animals (Figure 3). At 20 mM glucose, secretion was 3.92 ± 0.62 ng/islet/h from control islets, compared with 4.57 ± 0.76 ng/islet/h from islets of injected rats ($P < 0.05$, $N = 7$). The increase was not significant at basal (2 mM glucose) levels of secretion.

Islets from epicatechin-injected rats showed a higher insulin content than the controls (Figure 3). The amount of insulin per islet was increased from 126 ± 8.2 ng in the controls to 173.0 ± 22.1 ng ($P < 0.05$, $N = 9$). In addition, the proportion of insulin in terms of total islet protein rose from 48.22 ± 10.11 to 59.73 ± 11.77 ng/μg protein ($P < 0.05$, $N = 9$). Incidentally, the total protein content of the islets fell from 2.92 ± 0.42 μg in the controls to 2.67 ± 0.25 μg in the test group (Figure 3); this decrease was not significant.

The effects of epicatechin and glucose on secretion from islets cultured with epicatechin is shown in Table 2. After 4 days' culture in media containing 5.5 or 20 mM glucose, with or without 0.05 mM (−)epicatechin, islets were then incubated in low- and high-glucose media for 1 h at 37°C. Culturing of islets in low-glucose media significantly depressed insulin secretion in response to 20 mM glucose on subsequent testing ($P < 0.05$). Culturing in high-glucose medium resulted in a greater secretion from both glucose-stimulated and -unstimulated islets than from those cultured in low-glucose medium ($P < 0.05$, $N = 7$ in both cases). However, secretion from islets cultured in the presence of (−)epicatechin at either low- or high-glucose concentrations was not significantly increased when compared with control islets.

Table 3 shows the effects of epicatechin and glucose on DNA synthesis in islets from adult rats in culture. The addition of 0.05 mM epicatechin to the culture media resulted in an increase in DNA synthesis as shown by an increase in ^{3}H-thymidine incorporation, expressed as cpm/μg DNA.

The addition of 12.5 mM hydroxyurea to the culture medium significantly inhibited DNA synthesis in both the control islets and islets exposed to epicatechin. Culturing in medium containing 20 mM glucose did not result in a significant

TABLE 2
Effect of glucose and epicatechin on secretion of cultured islets

Conditions	Glucose concentration (mM) in static incubations	Secretion (ng/islet/h)	
		Control	Cultured in epicatechin
Freshly prepared islets	2	0.52 ± 0.09 †	
	20	4.30 ± 0.82 †	
Cultured at 5.5 mM glucose	2	0.14 ± 0.02 *	0.19 ± 0.03 *
	20	0.30 ± 0.06 *	0.44 ± 0.10 *
Cultured at 20 mM glucose	2	0.58 ± 0.11 *	0.76 ± 0.14
	20	1.15 ± 0.14 *	1.26 ± 0.46

Islets were cultured at 5.5 or 20 mM glucose, with or without 0.05 mM epicatechin, for 4 days. They were harvested for 1-h static incubation in the presence of 2 or 20 mM glucose. Details of experimental procedures are in MATERIALS AND METHODS. Values are means ± SEM, $N = 7$. Significance of differences between means of secretion from cultured islets: *$P < 0.05$. Significance of differences between means of secretion from freshly prepared islets: †$P < 0.01$.

TABLE 3
Effects of epicatechin on DNA synthesis

Glucose conc. of culture medium (mM)	^{3}H-thymidine labeling (cpm/μg DNA)			
	Control	Control + hydroxyurea	Epicatechin	Epicatechin + hydroxyurea
5.5	368.2 ± 52 (7)	113 ± 22 (3)†	549.5 ± 56 (7)*	149 ± 36 (3)†
20	423 ± 45 (7)		569 ± 74 (7)	

Islets were cultured for 4 days at 5.5 mM or 20 mM glucose, with or without 0.05 mM epicatechin. They were then replated for thymidine labeling for 24 h. Details of culturing, labeling, and counting are given in MATERIALS AND METHODS. Values are given as mean ± SEM. Significance of difference between controls and islets cultured in epicatechin: *$P < 0.05$; significance of difference between hydroxyurea-treated and -untreated islets: †$P < 0.01$. Numbers in parentheses are numbers of observations.

increase in total DNA synthesis in either test or control islets after 24 h of labeling.

DISCUSSION

Chakrawarthy et al.[1] reported that epicatechin injected intraperitoneally into albino rats that had previously been made diabetic with alloxan brought about a normalization of blood glucose levels together with B-cell regeneration. When injected into normal rats for 4 days, it protected their islets from the actions of alloxan. At the same time, the authors reported no signs of acute toxicity of the flavonoid, even in fairly large doses of up to 1 g/kg body wt when administered intraperitoneally to their animals. Our findings suggest that epicatechin can certainly have direct effects on the islets of Langerhans in vitro, and that these may have been, at least in part, responsible for the actions reported previously. Thus, it is clear that epicatechin increased insulin secretion from isolated rat islets of Langerhans during short-term, static incubations and in perifusion. The effect was ATP-, temperature-, and dose-dependent, and was therefore likely to be a true effect on secretion. Injection of epicatechin into normal rats brought about an increase in insulin secretion from isolated islets when exposed to 20 mM glucose, and a rise in islet insulin content (presumably reflecting an increase in insulin biosynthesis) in treated rats. DNA synthesis increased in islets cultured for 4 days in the presence of epicatechin. These results suggest the possibility that the reported B-cell regeneration brought about by epicatechin injection after alloxan treatment may be a direct effect on B-cell replication. The inhibition of ^{3}H-thymidine incorporation by hydroxyurea suggested that the predominant part of the radioactivity recovered from the cultured islets was incorporated in newly synthesized DNA.

The absence of an increase of the total protein content could depend on the small number of new cells produced in adult islets.[13 16] Under maximal glucose stimulation, the fraction of new cells formed over 24 h is < 3% of the total islet-cell population.[17] Another possible reason is that newly replicated B-cells may be smaller than pre-existing ones, so that together with the overall low rate of cell division in adult B-cells, an increase in total islet protein content could not be detected during the period of study.

It has been reported[18] that flavonoids inhibited cAMP phosphodiesterase and that epicatechin specifically inhibited the enzyme extracted from cardiac muscle.[19] Available evidence strongly suggests that cAMP may be a modulator of insulin secretion.[20,21] Despite the controversy over the role of cyclic AMP in the regulation of insulin biosynthesis[22–29] and islet cell replication,[26,30–33] it certainly seems possible that at least some of the effects of epicatechin on islet function that we have observed may be exerted via the cAMP system. Experiments to test this hypothesis are in progress, along with attempts to evaluate the potency and specificity of epicatechin as an insulin secretagogue and inducer of islet-cell replication in vivo.

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