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Choline increases serum insulin in rat when injected intraperitoneally and augments basal and stimulated aceylcholine release from the rat minced pancreas *in vitro*

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Intraperitoneal injection of choline (30–90 mg·kg^{-1}) produced a dose-dependent increase in serum insulin, glucose and choline levels in rats. The increase in serum insulin induced by choline (90 mg·kg^{-1}) was blocked by pretreatment with the muscarinic acetylcholine receptor antagonists, atropine (2 mg·kg^{-1}), pirenzepine (2 mg·kg^{-1}) and 4-diphenylacetoxy-*N*-methylpiperidine (2 mg·kg^{-1}) or the ganglionic nicotinic receptor antagonist, hexamethonium (15 mg·kg^{-1}). The effect of choline on serum insulin and glucose was enhanced by oral glucose administration (3 g·kg^{-1}). Choline administration was associated with a significant ($P < 0.001$) increase in the acetylcholine content of pancreatic tissue. Choline (10–130 μM) increased basal and stimulated acetylcholine release but failed to evoke insulin release from the minced pancreas at considerably higher concentrations (0.1–10 mM). Hemicholium-3, a choline uptake inhibitor, attenuated the increase in acetylcholine release induced by choline augmentation. Choline (1–32 mM) inhibited [^{3}H]quinuclidinyl benzilate binding to the muscarinic receptors in the pancreatic homogenates. These data show that choline, a precursor of the neurotransmitter acetylcholine, increases serum insulin by indirectly stimulating peripheral acetylcholine receptors through the enhancement of acetylcholine synthesis and release.

Keywords: precursor; acetylcholine; parasympathetic; muscarinic receptors; nicotinic receptor.

The availability of choline, the precursor of the neurotransmitter acetylcholine, is an important factor in the regulation of cholinergic neurotransmission. *In vitro* studies measuring acetylcholine synthesis and release in the presence of various concentrations of choline in the superfusion, perfusion or incubation media show that choline enhances acetylcholine synthesis and release during increased neuronal demand [1–5]. Treatments that increase circulating and tissue choline levels enhance acetylcholine efflux [6–9] and augment cholinergic transmission [10–12]. The dependency of cholinergic neurons on choline becomes more evident when the firing rate of neurons increases [2,3,5,7–9]. Under such conditions, administration of choline can greatly enhance cholinergic transmission [12]. In addition, choline produces biological effects at high concentrations by acting directly on acetylcholine receptors as an agonist [13]. Experimental studies conducted in our laboratory have demonstrated that central administration of choline to rats produces a variety of pharmacological actions; it increases blood pressure [14,15], induces hypothermia [16] and elevates plasma prolactin [17], corticotropin [18], β-endorphin [18], oxytocin [19] and vasopressin [20] concentrations. In humans, choline-containing compounds are reported to have some degree of therapeutic benefit in memory loss, ischemic and traumatic central nervous system injuries, aging and Alzheimer's disease [21–26].

Compared to reports on choline's effects in the central nervous system, relatively little is known about the effect of choline administration on the functions of peripheral organs. Evidence has accumulated over the years that acetylcholine acts a neurotransmitter in the neural control of insulin release (reviewed in [27–29]). The pancreatic islets are densely innervated by postganglionic cholinergic nerve fibers emanating from nerve cell bodies in the pancreatic ganglia that are innervated by the vagus nerves [27–30]. Cholinergic receptors have been observed in close association with insulin-secreting β-cells within pancreatic islets of several species [27–29,31,32]. Electrical stimulation of the vagus nerve or activation of cholinergic receptors by acetylcholine or muscarinic receptor agonists stimulates insulin secretion [31–39]. Participation of central cholinergic neurons and the vagus nerve in central nervous system-mediated neural regulation of insulin secretion from pancreas has also been demonstrated [40].

In agreement with the augmentation of cholinergic neurotransmission by choline and the role of cholinergic neurons in the control of insulin release, we recently observed that intraperitoneal choline administration increases serum insulin levels [41]. The present study was undertaken to characterize fully the mechanism responsible for the increase in serum insulin levels produced by choline administration in conscious rats. The objectives of this study

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Abbreviations: 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; QNB, quinuclidinyl benzilate; HC-3, hemicholinium-3.

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were to: (a) determine the dose–response relationship and the time course of the serum insulin response to choline; (b) determine whether the blockade of peripheral muscarinic and/or nicotinic cholinergic receptors inhibits the effect of choline on serum insulin levels; (c) determine whether oral glucose administration influences the response of serum insulin to choline; and (d) determine whether the increase in serum insulin levels produced by choline is mediated by a presynaptic and/or a postsynaptic mechanism.

Materials and methods

Animals

Male Wistar rats (Experimental Animals Breeding and Research Center, Uludag University Medical Faculty, Bursa,Turkey) weighing 300–350 g were used in all experiments. The animals were fed a standard pellet diet and tap water *ad libitum* and were exposed to a 12-h light-dark cycle. The experimental protocol was approved by the Animal Care and Use Committee of Uludag University (Bursa, Turkey), and all experiments conformed to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Experiments

For the dose and time-course studies, 28 rats were anesthetized with ether and a PE 50 cannula was inserted into the left carotid artery. After allowing recovering for 3 h, rats were injected intraperitoneally (i.p) with saline (1 mL·kg^{-1}) or choline chloride (30, 60 or 90 mg·kg^{-1}).

In experiment 2, rats were pretreated i.p. with either saline (1 mL·kg^{-1}), atropine methylnitrate (2 mg·kg^{-1}) or hexamethonium (15 mg·kg^{-1}) 10 min before i.p. injection of choline chloride (90 mg·kg^{-1}). In a related experiment, rats were pretreated i.p. with either saline (1 mL·kg^{-1}), pirenzepine (2 mg·kg^{-1}), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP; 2 mg·kg^{-1}) or AF-DX 116 (2 mg·kg^{-1}) 10 min before i.p. injection of choline chloride (90 mg·kg^{-1}).

In experiment 3, rats fasted overnight were injected i.p. with saline or choline (90 mg·kg^{-1}); 2 min later they were given either tap water (3 mL·kg^{-1}) or D-glucose (3 g·kg^{-1}) by gavage.

In experiment 4, rats received i.p. saline or choline chloride (90 mg·kg^{-1}); they were killed by rapid decapitation 10 min later. The pancreas was removed for acetylcholine measurement.

In experiment 5, the effects of various concentrations of choline on acetylcholine and insulin release from minced pancreas and on [^{3}H]quinuclidinyl benzilate (QNB) binding to pancreatic muscarinic acetylcholine receptors were investigated.

Chemical determinations

In the dose and time-course studies, a blood sample (0.1 mL) was withdrawn from an arterial catheter immediately before and 5, 10, 20, 30, 45 and 60 min after i.p. choline injection for serum insulin, glucose and choline measurements. In experiments 2 and 3, rats were killed by rapid decapitation 10 min after choline administration and trunk blood was collected for serum insulin and glucose measurements. Serum was separated by centrifugation and stored at −20 °C until assayed for insulin and choline. Serum glucose was assayed on the same day, within 60 min after the experiment was completed.

Serum insulin was determined by radioimmunoassay using a commercially available radioimmunoassay kit specific for rat insulin (Amersham Pharmacia Biotech, Buckinghamshire, England). The radioimmunoassay kit reliably detected 10–5000 pg of rat insulin per assay tube and 20–50 μL of serum was used for each analysis. The intra- and interassay coefficients of variability for the rat insulin assay were about 13 and 6%, respectively. The cross-reactivity of the rat insulin antibody with rat pancreatic polypeptide, rat pancreastatin or somatostatin was less than 0.01%.

Serum glucose levels were measured in 5 μL serum with the glucose oxidase method using a commercially available assay kit (Biotrol, France).

Serum choline levels were determined in 10 μL serum radioenzymatically [42]. In brief, choline was phosphorylated by choline kinase in the presence of [γ-^{32}P]ATP and isotopically labeled phosphocholine was separated from excess [γ-^{32}P]ATP and quantitated.

To measure tissue acetylcholine content, the pancreas was rapidly removed, weighed and homogenized in 5 mL of 1 M formic acid in acetone (15 : 85; v/v) by using an Ultra-Turrax tissue homogenizer. The homogenate was allowed to stand for about 24 h in a cold room and then centrifuged (1500 ***g*** for 15 min at 4 °C). The supernatant was transferred to a glass tube (13 × 100 mm) and dried under vacuum. The residue was dissolved in 2 mL of cold water and acetylcholine was then extracted into 10% (v/v) 2-butanone containing 1 mL 0.03 M HCl using a silica column procedure [43]. The acetylcholine containing column fraction was dried under vacuum, and the acetylcholine content of the dried samples was determined by the radioenzymatic method [44]. Standards for acetylcholine (0–2 μmol) were prepared in 5 mL of 1 M formic acid in acetone (15 : 85, v/v) and processed in parallel with the samples.

The effect of choline on *in vitro* release of acetylcholine from the pancreas was determined using minced pancreas (1 × 1 mm), prepared with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Gomstall, UK) and collected in cold medium. Minced pancreas was washed three times to remove most of the debris and then transferred into a superfusion chamber. The chamber was kept at 37 °C in a water bath, and the mince perfused with Krebs-Ringer buffer (120 mM NaCl, 3.5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM glucose and 0.02 mM eserine salicylate) for 60 min at a constant flow rate (0.6 mL·min^{-1}) using a peristaltic pump (Rainin Instrument Co, Woburn, MA, USA). This solution was bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Following the 60-min equilibration period, the minced pancreas was perfused for 2 h with a choline-free or choline (10–130 μM)-containing medium. Throughout this 2-h period, the minced pancreas were either maintained at rest (perfused with normal Krebs-Ringer buffer) or stimulated by high potassium (the KCl concentration was elevated from 3.5

to 50.0 mM and NaCl concentration was reduced from 120 mM to 73.5 mM in the perfusion buffer). The perfusate representing the entire 2-h rest or stimulation period was collected and assayed for acetylcholine and choline. In a related study, the minced pancreas were perfused for 2 h with choline-free or choline (10–130 μM)-containing medium in the presence or absence of hemicholinium-3 (HC-3; 20 μM). The minced pancreas was maintained at rest (perfused with normal Krebs-Ringer buffer) during first 60 min of this 2-h period and then stimulated for 60 min with high potassium (50 mM) medium. Perfusates from the entire 60 min rest or stimulation periods were collected and assayed for acetylcholine.

Acetylcholine and choline were extracted from the superfusate by a silica column procedure [43]. Two millilitres of the superfusate were applied to silica column (5 × 8 mm bed of Bio-Sil A, 200-400 mesh, Bio-Rad Laboratories, CA, USA). The column was then washed successively with 1 mL of 0.001 M HCl, 0.8 mL of 0.075 M HCl and 1 mL of 0.03 M HCl in 10% (v/v) 2-butanone. The latter fraction (0.03 M HCl in 10% 2-butanone) was collected in glass tubes (12 × 75 mm) and dried under vacuum. The dried samples were resuspended in 0.1 mL of Tris buffer (50 mM; pH = 8.0) containing 0.5 units of choline oxidase (Sigma Chem Co., St Louis, MO, USA) and 20 mM $MgCl_2$ and incubated for 30 min to remove excess choline. After the incubation period, 1 mL of 0.001 M HCl was added to the tube and the column procedure was repeated once again. The recovery of acetylcholine in the final HCl/butanone fraction was about 50%, and that of choline was less than 1%. These final fractions were dried under vacuum and assayed for acetylcholine by the radioenzymatic method [44]. Acetylcholine standards (0–2 μmol) were prepared in 2 mL of the same choline-containing medium as that used for superfusing the minced pancreas, and processed in parallel with the samples.

In vitro insulin release was determined using minced pancreas, prepared as described above and perfused with the same buffer, but without eserine, for 60 min. After the 60-min equlibration period, ≈100 mg of the minced pancreas were transferred into each of six incubation wells. Minced pancreas were incubated in 2 mL of Krebs-Ringer buffer containing 3% bovine serum albumin for 20 min; incubation media were replaced by 2 mL of buffer (37 °C) every 5 min. After the 20-min incubation period, the minced pancreas was incubated for an additional 10 min in 2 mL of buffer. The incubation medium was carefully removed for measurement of basal insulin release in the absence of added choline. The minced pancreas was then incubated again for six more 10-min periods in 2 mL buffer solutions containing increasing (from 0.1 mM to 10 mM) concentrations of choline. At the end of each 10-min incubation period, media were removed for measurement of insulin release. The insulin content of the incubation medium was measured by using 50 μL aliquots of the medium, using same radioimmunoassay kit described above for serum insulin measurement. Insulin release at each choline concentration was expressed as the percentage of insulin release during the first 10-min incubation period in the absence of added choline.

The muscarinic receptor binding activity of choline to the pancreatic homogenate was examined using [^{3}H]QNB (76 Ci·mmol^{-1}, Amersham Pharmacia Biotech, Buckimhamshire, England) as described previously [13]. Briefly, the pancreas was homogenized in 50 mM sodium/potassium phosphate buffer (pH = 7.4; 20 mg tissue per mL) using an Ultra-Turrax tissue homogenizer. The homogenate was filtered through three layers of cheesecloth and used fresh for receptor binding. Saturable [^{3}H]QNB binding was determined by incubating 1.8 mL of the homogenate at room temperature with increasing concentrations (0.1–6.4 nM) of the radioligand in a final volume of 2.0 mL of sodium/potassium phosphate buffer. The incubation was terminated after 60 min by vacuum filtration through Whatman GF/B filters. The filters were washed two times with 4 mL of ice-cold buffer and placed in a scintillation vial containing 4 mL of Aquasol-2 (New England Nuclear, Boston, MA, USA). Radioactivity was determined at least 3 h later in a Packard liquid scintillation counter. Specific binding was taken as that portion of total binding inhibited by 10 μM atropine. [^{3}H]QNB binding was expressed as pmol per mg tissue. For inhibition experiments, 1.8 mL of the pancreatic homogenate was incubated for 60 min at room temperature with a fixed concentration (0.9 nM) of [^{3}H]QNB and various concentrations of choline (0.1 mM to 32 mM). [^{3}H]QNB binding at each choline concentration was expressed as the percentage of specific binding in the absence added choline. An IC_{50} value for choline, the concentration of choline to produced 50% inhibition in the [^{3}H]QNB binding, was determined graphically by log-probit analysis.

Drugs

The following drugs were used: choline chloride, atropine methylnitrate, pirenzepine hydrochloride, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP methiodide), hexamethonium hydrochloride and HC-3 (Sigma Chemical Co., St Louis, MO, USA), and were dissolved in physiological saline (0.9% NaCl). The volume of solution injected i.p. was 1 mL·kg^{-1}.

Statistics

Data are presented as mean ± SEM. Statistical evaluation consisted of one- or two-way ANOVA followed by Tukey's test. Values of P less than 0.05 were considered to be significant.

Results

Effect of choline on serum insulin, glucose and choline levels

Figure 1 illustrates the changes in serum insulin, glucose and choline levels, for a period of 60 min, following i.p. injection of choline (30, 60 or 90 mg·kg^{-1}) or saline (1 mL·kg^{-1}). Serum insulin and glucose levels began to increase within 5 min after choline injection, reached a maximum within 10 min and returned to baseline levels by 30–45 min, depending upon the dose. The increases in serum insulin, glucose and choline induced by choline were time- and dose-dependent.

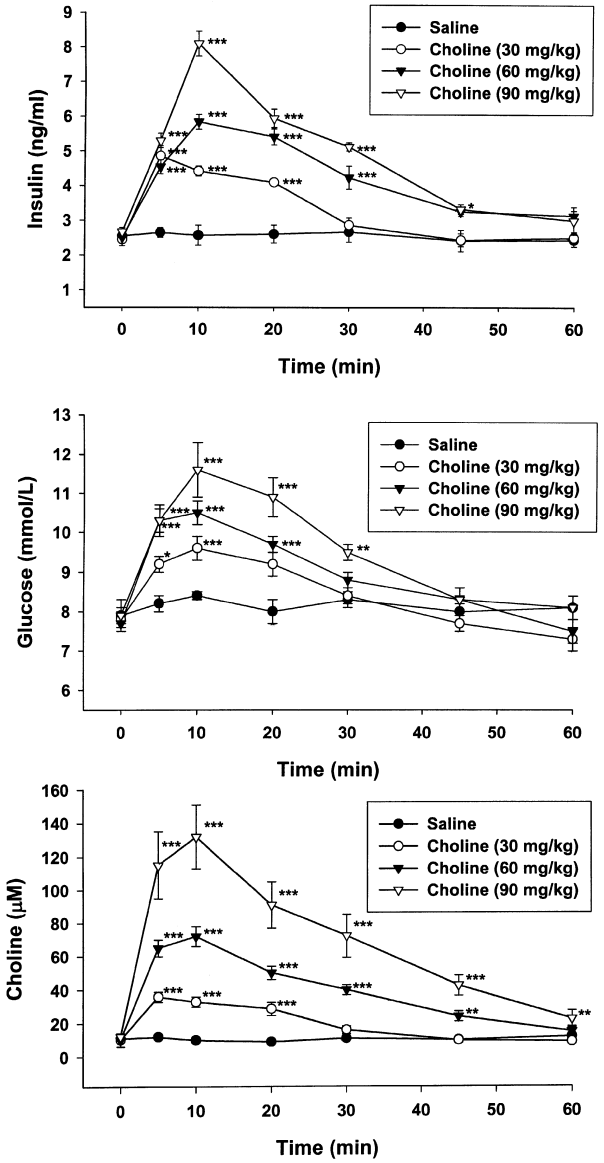

Fig. 1. Choline administration increases serum insulin (top), glucose (middle) and choline (bottom) concentrations. Rats were injected i.p. with saline (1 mL·kg^{-1}) or choline chloride (30, 60 or 90 mg·kg^{-1}). Blood samples (0.1 mL) were collected through a catheter inserted into the left carotid artery, immediately before (0) and 5, 10, 20, 30, 45 and 60 min after treatment. Each point represents the mean ± SEM of seven measurements. Data were analyzed with two-way ANOVA with repeated measures followed by Tukey's test. *P < 0.05; **P < 0.01; ***P < 0.001 compared with the same time point from saline treated controls.

Effects of muscarinic and nicotinic receptor antagonists on choline-induced increases in serum insulin

Rats were pretreated i.p. with saline (1 mL·kg^{-1}), atropine methylnitrate (2 mg·kg^{-1}) or hexamethonium (15 mg·kg^{-1}) 10 min prior to saline or choline injection (90 mg·kg^{-1}; i.p.). Pre-treatment with hexamethonium, a peripherally acting selective antagonist of the ganglionic nicotinic receptor, entirely blocked the choline-induced increase in serum insulin (Table 1). Pre-treatment with atropine methylnitrate, a peripherally acting nonselective muscarinic receptor antagonist, also blocked the choline-induced increase in insulin (Table 1).

Table 1. Effects of atropine methylnitrate and hexamethonium chloride on the increases in serum insulin elicited by i.p. choline. Rats were pretreated i.p. with saline (1 mL·kg^{-1}) atropine methylnitrate (2 mg·kg^{-1}) or hexamethonium chloride (15 mg·kg^{-1}) 10 min before i.p. administration of saline (1 mL·kg^{-1}) or choline (90 mg·kg^{-1}). The animals were sacrificed 10 min after the second i.p. injection and blood samples were collected for serum insulin measurements. Data are expressed as the mean ± SEM (n = 7). Data were analyzed by two-way ANOVA followed by Tukey's test. *P < 0.001 compared with the values from saline control.

Pretreatment + treatment	Insulin (ng·mL^{-1})
Saline + saline	2.5 ± 0.2
Saline + choline	6.1 ± 0.4*
Atropine methylnitrate + saline	3.3 ± 0.4
Atropine methylnitrate + choline	3.6 ± 0.4
Hexamethonium + saline	2.6 ± 0.1
Hexamethonium + choline	2.6 ± 0.3

Effects of relatively selective antagonists of muscarinic receptors on choline-induced increases in serum insulin

To determine the involvement of muscarinic acetylcholine receptor subtypes in the increase in serum insulin elicited by choline, rats were pretreated i.p. with saline (1 mL·kg^{-1}), pirenzepine (2 mg·kg^{-1}), 4-DAMP (2 mg·kg^{-1}) or AF-DX 116 (2 mg·kg^{-1}) 10 min prior to choline injection (90 mg·kg^{-1}; i.p). The increase in serum insulin induced by choline was blocked by pirenzepine, a relatively selective antagonist of M_1 muscarinic receptors [45], and 4-DAMP, a relatively selective antagonist of M_1 + M_3 receptors [45] (Table 2). Pre-treatment with AF-DX 116, a relatively

Table 2. Effects of relatively selective antagonists of muscarinic receptor subtypes on the increase in serum insulin induced by i.p. choline. Rats were pretreated i.p. with saline (1 mL·kg^{-1}), pirenzepine (2 mg·kg^{-1}), 4-DAMP (2 mg·kg^{-1}) or AF-DX 116 (2 mg·kg^{-1}) 10 min before i.p. administration of saline (1 mL·kg^{-1}) or choline (90 mg·kg^{-1}). The animals were sacrificed 10 min after the second i.p. injection and blood samples were collected for serum insulin measurement. Data are expressed as the mean ± SEM (n = 6 or 7). Data were analyzed by two-way ANOVA and followed by Tukey's test. *P < 0.001 compared with the values from saline control. **P < 0.01 compared with the values from 'saline + choline' group.

Groups	Insulin (ng·mL^{-1})
Saline + saline	2.8 ± 0.6
Saline + choline	5.8 ± 0.6*
Pirenzepine + saline	2.4 ± 0.3
Pirenzepine + choline	3.1 ± 0.4
4-DAMP + saline	2.6 ± 0.3
4-DAMP + choline	3.2 ± 0.3
AF-DX 116 + saline	3.5 ± 0.6
AF-DX 116 + choline	8.5 ± 0.8**

selective antagonist of M_2 receptors [45], enhanced the increase in serum insulin induced by i.p. choline (Table 2).

Effects of oral glucose on serum insulin and glucose responses to choline

To determine whether oral glucose administration alters the increases in serum insulin and glucose evoked by choline (90 mg·kg^{-1}), rats were fasted overnight and given either water (5 mL·kg^{-1}) or water containing glucose (3 g per kg per 5 mL) orally by stomach tube two min after i.p. administration of saline or choline (90 mg·kg^{-1}). Oral administration of glucose (3 g·kg^{-1}) resulted in a significant rise in serum insulin and glucose levels in both i.p. saline or choline-treated rats (Table 3). In choline treated animals, the increases in serum insulin and glucose were much higher than the control animals. Analysis of variance revealed a significant effect of choline ($F_{1,24} = 26.51$; $P < 0.001$), glucose ($F_{1,24} = 76.84$; $P < 0.001$) and a significant interaction between choline and glucose ($F_{1,24} = 12.84$; $P < 0.001$) on serum insulin. Similarly, there were significant effects of choline ($F_{1,24} = 32.95$; $P < 0.001$), glucose ($F_{1,24} = 168.28$; $P < 0.001$) and a significant interaction between choline and glucose ($F_{1,24} = 10.66$; $P < 0.003$) on serum glucose levels.

Effect of choline on acetylcholine levels in the pancreas

Acetylcholine levels in the pancreas were 2.8 ± 0.2 nmol·g^{-1} tissue ($n = 9$) and 4.1 ± 0.3 nmol·g^{-1} tissue ($n = 9$; $P < 0.005$) 10 min after i.p. injection of saline or choline (90 mg·kg^{-1}), respectively. Statistical analysis of the data (Mann–Whitney Rank Sum Test) revealed a significant ($P < 0.01$) effect of choline on acetylcholine levels in the pancreas.

Effect of choline on acetylcholine release from minced pancreas

Minced pancreas released acetylcholine and choline into the medium at rest and during superfusion with a choline-free high potassium medium. The rate of choline released from the minced pancreas was 5.5 ± 1.0 nmol·min^{-1}·g^{-1} tissue ($n = 6$) at rest or 8.5 ± 1.5 nmol·min^{-1}·g^{-1} tissue ($n = 6$) during 2-h stimulation period with high potassium (50 mM) medium, respectively. In the absence of added exogenous choline, the total amount of acetylcholine released during 2 h perfusion with normal or high potassium Krebs-Ringer medium was 4.8 ± 1.0 nmol per 2 h per g tissue ($n = 6$) and 12.6 ± 1.8 nmol per 2 h per g tissue ($n = 12$), respectively. The amount of acetylcholine released into the medium rose after addition of exogenous choline to the superfusion medium (Fig. 2). One-way analysis of variance revealed a significant concentration effect of choline on basal ($F_{3,20} = 23.91$; $P < 0.001$) and stimulated ($F_{5,40} = 55.45$; $P < 0.001$) acetylcholine release from the minced pancreas.

Effect of HC-3 on choline-induced increases in acetylcholine release from the minced pancreas

To determine whether choline-induced increases in acetylcholine release are sensitive to HC-3, a selective inhibitor of the high-affinity choline uptake system [4,46,47], acetylcholine release from minced pancreas was tested in the presence or absence of HC-3 in the perfusion medium. As seen in Fig. 3, the presence of HC-3 (20 μM) in the medium blocked the increase in basal (Fig. 3, top) or stimulated (Fig. 3, bottom) acetylcholine release from the minced pancreas induced by 40 μM choline. HC-3 greatly attenuated, but failed to block, the increases in basal and stimulated acetylcholine release induced by 65 or 130 μM of choline (Fig. 3). Analysis of variance revealed a significant effect of HC-3 ($F_{1,40} = 45.05$; $P < 0.001$), concentration of choline ($F_{3,40} = 101.76$; $P < 0.001$) and a significant interaction

Table 3. Effects of oral glucose administration on the increases in serum insulin and glucose elicited by i.p. choline. Fasted rats (for 20–21 h) were injected i.p. with saline (1 mL·kg^{-1}) or choline (90 mg·kg^{-1}); 2 min later they were treated orally either water (5 mL·kg^{-1}) or water containing glucose (3 g per kg per 5 mL). The animals were sacrificed 10 min after the i.p. injection of saline or choline and blood samples were collected for serum insulin and glucose measurements. Data are expressed as the mean ± SEM ($n = 8$ for each treatment). Data were analyzed by two-way ANOVA followed by Tukey's test. *$P < 0.05$; **$P < 0.001$ compared with the values from the 'saline + water' group. #$P < 0.001$ compared with the values from the 'choline + water' and 'saline + glucose' groups.

Groups	Insulin (ng·mL^{-1})	Glucose (mmol·L^{-1})
Saline + water	1.2 ± 0.2	6.2 ± 0.3
Choline + water	3.6 ± 0.5**	7.5 ± 0.3*
Saline + glucose	1.9 ± 0.2*	10.8 ± 0.3**
Choline + glucose	7.6 ± 0.7**, #	14.6 ± 1.1**, #

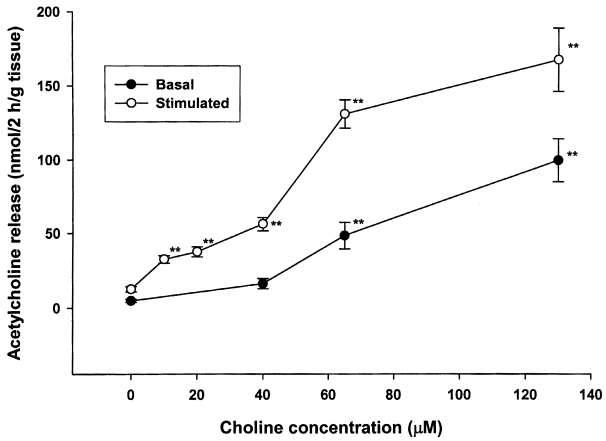

Fig. 2. Effects of choline on acetylcholine release from minced pancreas. The minced pancreas were perfused for 2 h with normal (basal) or high potassium (stimulated) Krebs-Ringer buffer containing the indicated concentration of choline (0–130 μM). The perfusates were collected and acetylcholine was extracted and measured radioenzymatically. Each point represents the mean ± SEM of six or eight measurements. Data were analyzed with one-way ANOVA followed by Tukey's test. *$P < 0.05$; **$P < 0.001$ compared with the values observed in the absence of exogenously added choline (0).

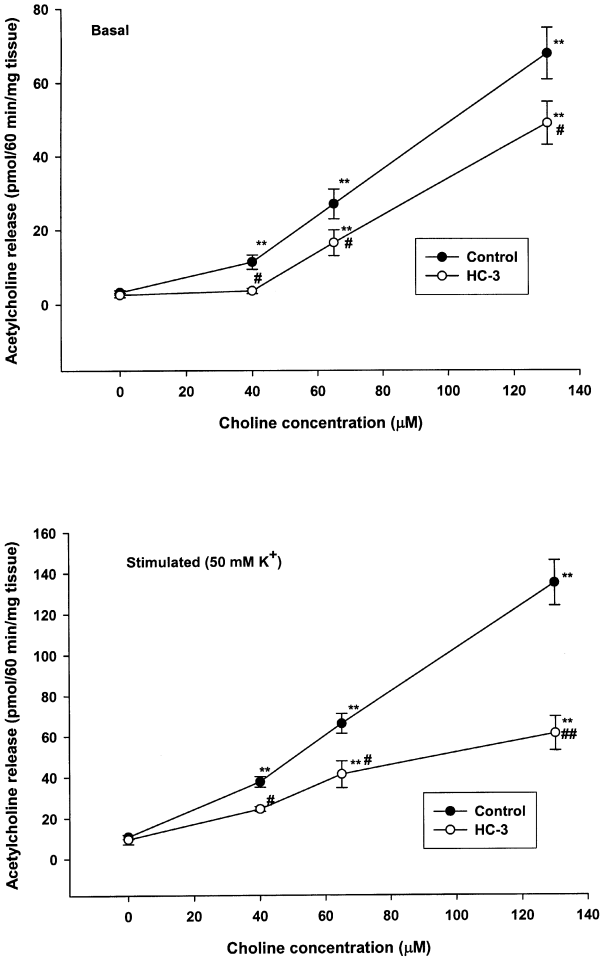

Fig. 3. Effect of HC-3 on the choline-induced increase in acetylcholine release from minced pancreas under basal and stimulated conditions. Minced pancreas were perfused for 1 h with the normal (basal) or high potassium (stimulated) Krebs-Ringer buffer containing the indicated concentration of choline (0–130 μM) for 1 h in the presence or absence of HC-3 (20 μM). The perfusates were collected and acetylcholine was extracted and measured radioenzymatically. Each point represents the mean ± SEM of six measurements. Data were analyzed with two-way ANOVA followed by Tukey's test. $*P < 0.05$; $**P < 0.001$ compared with the respective values observed in the absence of exogenously added choline (○). $\#P < 0.05$; $\#\#P < 0.001$ compared with corresponding values from the control.

between HC-3 and choline ($F_{3,40} = 17.68$; $P < 0.001$) on stimulated acetylcholine release.

Effect of choline on insulin release from minced pancreas

To determine if choline elicits insulin release *in vitro*, minced pancreas was incubated with various concentrations of choline. The rate of insulin release from the minced pancreas during incubation in absence of added exogenous choline into the medium was 270 ± 30 ng per 10 min per g tissue ($n = 8$). As seen in Fig. 4, addition of exogenous choline (100–10 000 μM) to the incubation medium failed to alter the rate of insulin release from the minced pancreas.

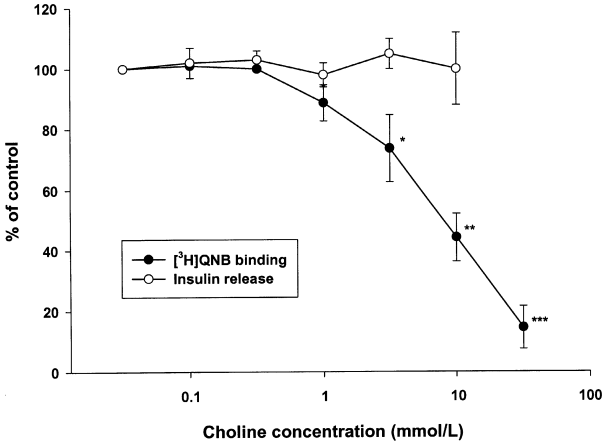

Fig. 4. Effects of choline on insulin release from minced pancreas and on [^{3}H]QNB binding in pancreatic homogenates. For release experiments, the minced pancreas were incubated in 2 mL of Krebs-Ringer buffer containing the indicated concentration of choline (0–10 mM) for 10 min. The incubation media were removed and insulin levels were analyzed by radioimmunoassay. Insulin release is presented as the percentage of the control release in the absence of added choline. The control insulin release was 270 ± 30 ng per 10 min per g tissue ($n = 8$). For binding experiments, membrane preparations of pancreas were incubated with [^{3}H]QNB (0.9 nM) for 60 min at room temperature with various concentrations of choline (0–32000 μM). [^{3}H]QNB binding at each choline concentration is expressed as the percentage of specific binding in the absence of added choline (the control binding). The control binding was 2.1 ± 0.2 pmol·g^{-1} tissue ($n = 6$). Each point represents the mean ± SEM of six or eight measurements. Data were analyzed with one-way ANOVA with repeated measures followed by Tukey's test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with the control values.

Effects of choline on [^{3}H]QNB binding to the pancreatic homogenate

The concentration of choline necessary to displace [^{3}H]QNB from muscarinic receptor binding sites was assessed by incubating the pancreatic homogenate with a fixed concentration of [^{3}H]QNB (0.9 nM) and various concentrations of choline (0.1–32 mM). A significant inhibition (13 ± 3%; $P < 0.05$) was first observed at a choline concentration of 1 mM (Fig. 4). The concentration of choline necessary to produce 50% inhibition of [^{3}H]QNB binding was 3.1 ± 0.6 μM ($n = 6$).

Discussion

In the present study we have shown that i.p. administration of choline to rats elevates serum insulin. Pretreatment with atropine methylnitrate, a peripheral muscarinic acetylcholine receptor antagonist, blocked the choline-induced increase in blood insulin. The increase in serum insulin elicited by choline was also prevented by pretreatment with the M1 antagonist, pirenzepine, or the M1 + M3 antagonist, 4-DAMP. Pretreatment with hexamethonium, an antagonist of ganglionic nicotinic acetylcholine receptors, prevented the choline-induced increase in serum insulin. Choline increased the acetylcholine content of the pancreas,

and enhanced acetylcholine release from minced pancreas, which suggests that choline stimulates insulin secretion indirectly by enhancing acetylcholine synthesis and release. In support of this conclusion, we found that choline failed to inhibit [^{3}H]QNB binding to pancreatic homogenates at concentrations that maximally stimulated insulin release although substantially higher choline concentrations did effectively displace [^{3}H]QNB.

Six decades ago, Sergeyeva [48] microscopically examined the pancreatic islets of cats after the administration of choline chloride and concluded that choline has a stimulatory effect on the β-cell. The present results are in accord with this very early finding, and also confirm and extend a recent report [41] from our laboratory showing that choline elevates blood concentrations of insulin. The observed increase in serum insulin levels after choline is also in good agreement with previous studies from other laboratories [33–39] showing that cholinergic stimulation causes insulin release and provides another example of the ability of choline to cause a neuroendocrine response that is cholinergic in nature.

Our finding that choline induced insulin release was blocked by both the muscarinic receptor antagonist atropine methyl nitrate and the nicotinic receptor antagonist hexamethonium indicates that the effect of choline is mediated by both muscarinic and nicotinic receptors. These results are in good accordance with previous studies demonstrating the involvement of both muscarinic [31–34,37] and nicotinic receptors [38,39] in the regulation of insulin release. It is known that insulin release from β-cells induced with cholinergic agonists is mediated mainly by muscarinic M_3 receptors [31,32,36,37,49]. In the present study, the increase in serum insulin induced by choline was enhanced by pretreatment with AF-DX 116, a relatively selective antagonist of the M_2 muscarinic receptor subtype [45], but was prevented by pirenzepine, a relatively selective M_1 antagonist, and 4-DAMP (Table 2), an antagonist of M_1 and M_3 muscarinic receptor subtypes [45]. These results suggest, but do not confirm, that M_1 and M_3 receptors may be involved in the increase in serum insulin induced by choline. Recently it has been reported [49] that M_2 and M_4 subtypes of muscarinic receptors mediate a paradoxical inhibitory effect on an insulin-secreting β cell line and blockade of M_2 subtypes by methoctramine enhances acetylcholine stimulated insulin secretion. The enhancement of serum insulin response to choline by AF-DX 116 is in agreement with these observations and suggests that choline induced insulin secretion may be inhibited by M_2 muscarinic receptor.

The release of insulin from the pancreas is stimulated by direct action of glucose on β-cells and this glucose induced insulin release is enhanced by cholinergic activation [36]. The enhancement by choline of serum insulin response to orally administered glucose (Table 3) was in agreement with this view [36]. However, the increase in serum insulin to i.p. choline was not due to the increase in serum glucose. Choline elevates serum insulin and glucose through different mechanisms. We demonstrated that the choline-induced hyperglycemia is mediated by nicotinic receptors, and the stimulation of catecholamine release from the adrenal gland and subsequent activation of α-adrenoceptors involves in the hyperglycemic response to choline [41].

The observed muscarinic and nicotinic actions of choline, as reflected by the increase in serum insulin, may thus result from its precursor effect on the pancreatic cholinergic neurons [1–5] and/or its direct effect on cholinergic receptors [13,50,51]. In the present study we observed that choline administration increased serum choline concentrations in a dose-dependent manner and that serum choline levels were maximally elevated from the basal level (about 10 μM) to 130–150 μM 10 min after the highest dose (90 mg·kg^{-1}) used in the present study (Fig. 1) which was associated with a significant increase (by about 1.5-fold) in pancreatic acetylcholine content. We also observed that choline, at concentrations of 10–130 μM, enhanced acetylcholine release from the minced pancreas in a concentration-dependent manner. The finding that HC-3 attenuated, but did not completely block (Fig. 3), the increase in acetylcholine release induced by choline augmentation suggests that choline taken up by both the high-affinity (HC-3 sensitive) and the low-affinity (HC-3 insensitive) transport systems may be a significant source of choline for acetylcholine synthesis in pancreatic cholinergic neurons like in the rat atrial tissue [4,47]. At much higher concentrations, 1–32 mM, choline interacted with the pancreatic muscarinic receptors (Fig. 4), but failed to induce insulin release from the minced pancreas (Fig. 4). Thus, in the present study serum choline concentrations achieved after choline administration were sufficient to increase pancreatic acetylcholine concentrations and acetylcholine synthesis and release in the pancreatic tissue (Figs 2 and 3), but were insufficient to interact with pancreatic muscarinic receptors (Fig. 4) or to evoke insulin release from the tissue (Fig. 4). We conclude therefore that choline elevates serum insulin by stimulating acetylcholine release, but not by activating muscarinic and/or nicotinic receptors directly in the pancreas.

In conclusion, the results from the present study show that choline elevates blood insulin by a peripheral mechanism. The effect of choline on serum insulin involves parasympathetic activation and is mediated both by peripheral muscarinic and nicotinic receptors. Insulin has several actions in the periphery as well as in the central nervous system. It is likely therefore that choline alters the functions in which insulin has a role, and that the increase in serum insulin can mediate some of the actions of choline.

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