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Cholinoceptor-mediated effects on glycerol output from human adipose tissue using *in situ* microdialysis

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1 Possible cholinoceptor-mediated effects on lipolysis were investigated *in vivo* in human subcutaneous adipose tissue of non-obese, non-smoking, healthy subjects, by use of microdialysis. Cholinomimetic and sympathomimetic agents were added to the ingoing dialysate solvent.

2 Addition of nicotine to the perfusion solvent caused a concentration-dependent reversible increase in the levels of glycerol in the dialysate (lipolysis index). The opposite effect (also concentration-dependent and reversible) was caused by the addition of carbachol. The maximum effects were 100% stimulation and 50% inhibition, respectively, by nicotine and carbachol. Neither nicotine nor carbachol stimulated nutritive blood flow in adipose tissue (as measured with an ethanol escape technique).

3 The nicotine effect *in situ* was concentration-dependently counteracted by the nicotinic cholinoceptor antagonist, mecamylamine. Likewise, the carbachol effect was concentration-dependently counteracted by the muscarinic cholinoceptor antagonist, atropine.

4 When adipose tissue was pretreated with phentolamine plus propranolol in order to obtain a complete α and β-adrenoceptor blockade, the subsequent addition of nicotine or carbachol still induced an increase and decrease in dialysate glycerol levels (lipolytic or antilipolytic effects), respectively. When adipose tissue was pretreated with mecamylamine or atropine, the subsequent addition of acetylcholine caused a reversible decrease and increase, respectively, of the dialysate glycerol levels.

5 Nicotine and carbachol had no effects on glycerol release from human isolated subcutaneous fat cells that were incubated *in vivo*.

6 In conclusion, the data demonstrate a dual effect of the cholinoceptor system on glycerol output in human adipose tissue: stimulation through nicotinic receptors and inhibition through muscarinic receptors. These effects, which are not observed *in vitro*, are independent of the adrenergic system and the local blood flow and seem not to be mediated by a direct action on the fat cell.

Keywords: Adipose tissue; acetylcholine; glycerol; blood flow; nicotinic receptors; muscarinic receptors

Introduction

Several body systems, such as exocrine glands, smooth muscle and heart muscle, are regulated by peripheral adrenoceptors, as well as cholinoceptors. It is generally believed, however, that adrenoceptors are solely involved in the regulation of metabolism (Guyton, 1986; Lefkowitz *et al.*, 1990; McIsaac, 1992). In skeletal muscle, where cholinoceptors as well as adrenoceptors are present, only the latter receptors are believed to influence glycogenolysis (Guyton, 1986; Lefkowitz *et al.*, 1990). Several unsuccessful attempts have been made to demonstrate a cholinergic regulation of lipolysis in adipose tissue, and it is widely assumed that cholinoceptors are not present in this tissue (Rosell & Belfrage, 1979; Fredholm, 1985). However, adrenoceptors are present in the fat cell mediating dual effects on lipolysis: inhibition via α_2-adrenoceptors and stimulation through β-adrenoceptor subtypes, as reviewed by Arner (1992).

Most of our knowledge about the cholinoceptor system is derived from studies on laboratory animals. Therefore, we have re-evaluated the existence of functional cholinoceptors in human adipose tissue by investigating lipolysis *in vivo*, with a new microdialysis technique. Microdialysis can be used to monitor lipolysis and the local nutritive blood flow continuously in human subcutaneous adipose tissue, as recently discussed in detail (Arner & Bülow, 1993). Small microdialysis probes are implanted in the extracellular space of adipose tissue and perfused with an ethanol-based dialysis solvent. The uptake of glycerol from the tissue by the dialysate, and the escape of ethanol into the tissue from the perfusate, are used as indices of lipolysis and nutritive flow, respectively. In addition, it is possible to deliver metabolic and/or vasoactive substances directly to the tissue through the probe. These agents only act locally, thus avoiding the influence of systemic effects of the administered substances.

We have microdialyzed human subcutaneous adipose tissue of healthy, non-obese and non-smoking subjects, with cholinomimetic and sympathomimetic agents at rest. Evidence for *in vivo* functional nicotinic and muscarinic cholinoceptors is presented.

Methods

Subjects

The experimental group was composed of 53 healthy, drug-free, non-obese and non-smoking subjects (26 men and 27 women). The ages ranged from 20 to 46 years (mean 30 years) and the body mass index (kg m^{-2}) ranged from 19 to 25 (mean 22). All subjects took regular moderate exercise, but none of them participated in athletics. The subjects were investigated on one or two occasions. In the experiments on isolated fat cells, subcutaneous adipose tissue was obtained from the abdominal area during elective surgery for non-malignant disorders, on drug-free subjects. They fasted over-night and only saline was given intravenously until the biopsy was taken (at the start of the operation). Anaesthesia was induced with a short-acting barbiturate and maintained with phentanyl in combination with a mixture of oxygen and nitrous oxide. These subjects were not selected on the basis of age, sex or body-weight. The study was approved by the Committee on

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Ethics at Karolinska Institute. The subjects were given a detailed description of the study and their consent was obtained.

Microdialysis probe

The microdialysis probe was supplied by CMA Microdialysis, Stockholm, Sweden. It has been described in detail elsewhere (Tossman & Ungerstedt, 1986). Briefly, a dialysis tube (10 × 0.5 mm, 20 000 mol. wt. cutoff) was glued to the end of a double-lumen steel cannula. The perfusion solvent enters the probe through the inner cannula, passes the dialysis membrane, and leaves the probe through the outer cannula, from which it is collected.

Experimental design

The experiments were performed at rest, as described in detail by Galitsky *et al.* (1993). Briefly, subjects were investigated in the supine position at 08 h 00 min after an overnight fast. The dialysis probe was inserted percutaneously without anaesthesia into the abdominal subcutaneous adipose tissue, immediately to the right of the umbilicus. Depending on the type of experiment, one to three probes were used on each occasion. The distance between each probe was always 30 mm. The inlet tubing of the probe was connected to a microinjection pump (CMA Microdialysis, Stockholm, Sweden), and was continuously perfused for 3.5 h with an ethanol-based (50 mM) Ringer solution, which was composed as described by Arner *et al.* (1991). Cholinomimetic and sympathomimetic agents were added in different combinations as sterile solutions to the ingoing perfusate. Nicotine, carbachol and acetylcholine were used as cholinomimetic agonists, and were present only for 1 h of the experiment (between 1.5 and 2.5 h), unless otherwise stated. Propranolol and phentolamine were used as β- and α-adrenoceptor-antagonists, repectively, and atropine and mecamylamine were used as muscarinic and nicotinic cholinoceptor antagonists, respectively. The antagonists were either absent or present throughout the whole 3.5 h-experimental period. In each experiment, fractions of the outgoing dialysate were collected for analysis of glycerol (Hellmér *et al.*, 1989) or ethanol (Bernt & Gutman, 1974). Ethanol was also determined in the ingoing perfusate and the ratio of ethanol leaving vs. ethanol entering was determined. Changes in this ratio reflect changes in the nutritive blood flow of the adipose tissue, as discussed in detail (Arner & Bülow, 1993). When adipose tissue was perfused with ethanol - Ringer solution alone, there was a constant glycerol level and ethanol ratio in the dialysate for at least 3 h (Galitsky *et al.*, 1993). The coefficients of variations for the baseline levels of glycerol and ethanol ratio were 10% and 5%, respectively. It has been demonstrated, that 50 mM ethanol does not alter lipolysis, and has no effects of its own on adipose tissue blood flow (Galitsky *et al.*, 1993). In all the nicotine experiments (which were performed during 1992), except those in Figure 4, the perfusion speed was 2.1 μl min^{-1} and the fraction collection time was 10 min. It was, for technical reasons, difficult to perform the experiments with this rapid collection time. Therefore a practical modification of the procedures was introduced in subsequent studies. In all the carbachol experiments and the nicotine experiments in Figure 5 (performed after 1992), the speed was 1.4 μl min^{-1} and fractions were collected every 15 min. Thus, the total sample volumes were identical on both occasions (21 μl). In additional experiments, lipolysis was investigated *in vitro*, as described in detail (Lönnqvist *et al.*, 1992). In brief, a subcutaneous fat biopsy was obtained. Isolated fat cells were prepared and incubated for 2 h at 37°C in diluted conditions (2%, v/V) in an albumin-containing buffer; the composition of the buffer has been described (Lönnqvist *et al.*, 1992). The following agents were added to the incubation medium: carbachol (1 – 100 μM), nicotine (1 μM – 1 mM) and noradrenaline bitartrate (ACO, Stockholm, Sweden; 1 nM – 1 μM). An aliquot of the medium was removed for determination of glycerol (Hellmér *et al.*, 1989).

Drugs

The following agents were added to the microdialysis solvent as sterile solutions: nicotine (Swedish Tobacco Group, Stockholm, Sweden), acetylcholine, mecamylamine and carbachol (Sigma Chemical Co., St Louise, MP, U.S.A.), (±)-propranolol (Pharmacia, Stockholm, Sweden), atropine (NM Pharma Co., Stockholm, Sweden) and phentolamine (Ciba-Geigy, Basel, Switzerland).

Data analysis

Values for glycerol are presented as a percentage of the mean glycerol levels during the first 0.5 h of microdialysis, which represents a pre-experimental baseline value. Thus, the experimental period in all cases, unless otherwise stated, was composed of a 1 h pretreatment period, a 1 h treatment period (nicotine, carbachol, or acetylcholine) and a 1 h post-treatment period. All statistical calculations were made on absolute values for glycerol, obtained during the 3 h mentioned above. These values were distributed in a normal fashion. Values for ethanol are presented as percentages of the concentration in the perfusion solvent, because these values mirror local nutritive blood flow, as discussed (Arner & Bülow, 1993). Values are means with the standard error of the mean (s.e.) indicated. Analysis of variance (ANOVA) was used for determination of the significance of the statistical comparisons, with Fisher PLSD test for post-hoc analysis. Since analysis of serial measurements was performed, data were also sometimes transformed to summary measures (i.e. area under the curve), before the statistical comparison as described (Matthews *et al.*, 1990). In some cases a linear regression analysis was also performed.

Results

Figure 1 shows the effect of nicotine (selective agonist for nicotine cholinoceptor subtypes) on dialysate glycerol levels. At rest, this ratio is almost constant for at least 3 h, as judged by recently microdialysis experiments with human adipose tissue (Galitsky *et al.*, 1993). Nicotine treatment caused a rapid, somewhat transient, but concentration-dependent (0.003 μM – 1 mM) increase in the glycerol levels ($P<0.01$ by ANOVA). When nicotine was removed from the perfusion solvent, the glycerol levels in the dialysate of the adipose tissue declined rapidly. At the highest nicotine concentration (1 mM), the glycerol concentration in the tissue dialysate was doubled, as compared to the baseline level. Pretreatment for 1 h with the selective nicotine receptor blocker mecamylamine (0.01 – 1 mM) prior to the nicotine (1 mM) infusion caused a significant concentration-dependent inhibition of the nicotine effect ($P<0.01$ by ANOVA), which was almost complete (90%) when equimolar concentrations (1 mM) of mecamylamine and nicotine were used (Figure 1). The basal levels of glycerol were not influenced by the mecamylamine (data not shown).

Figure 2 shows the effects of the muscarine-like cholinoceptor agonist, carbachol, on the dialysate glycerol levels. Carbachol caused a rapid, concentration-dependent (1 μM – 1 mM) and sustained decrease in the dialysate glycerol levels ($P<0.01$ by ANOVA), which gradually returned towards baseline; carbachol was then removed from the perfusion medium. The maximum effect was about 50% decrease in the dialysate glycerol levels, at 1 mM carbachol. Addition of the selective muscarinic receptor antagonist, atropine (0.1 – 10 μM), to the perfusion solvent counteracted in a concentration-dependent manner the glycerol-lowering effect of carbachol ($P<0.05$ by ANOVA). Atropine (10 μM) completely counteracted the effects of 10μM carbachol. Atropine did not influence basal glycerol levels (data not shown).

In order to study a possible effect of nicotine and carbachol on local adipose tissue blood flow, ethanol was included in the

microdialysis perfusion medium, and the escape of alcohol from the dialysate of adipose tissue was measured as the ratio of the outgoing versus the ingoing ethanol concentrations. At rest, this ratio is almost constant for at least 3 h, as judged by recent microdialysis experiments with human adipose tissue (Galitsky *et al.*, 1993). As shown in Figure 3, neither nicotine (1 mM) nor carbachol (0.01 mM) changed the ethanol ratio. Carbachol also did not alter local blood flow when the agent was added at 1 mM (data not shown). These data suggest that neither carbachol nor nicotine, at the highest concentrations used, influenced local adipose tissue blood flow under the present experimental conditions. When mecamylamine or atropine were added to the control dialysis solvent during the second hour of microdialysis, there were no significant changes

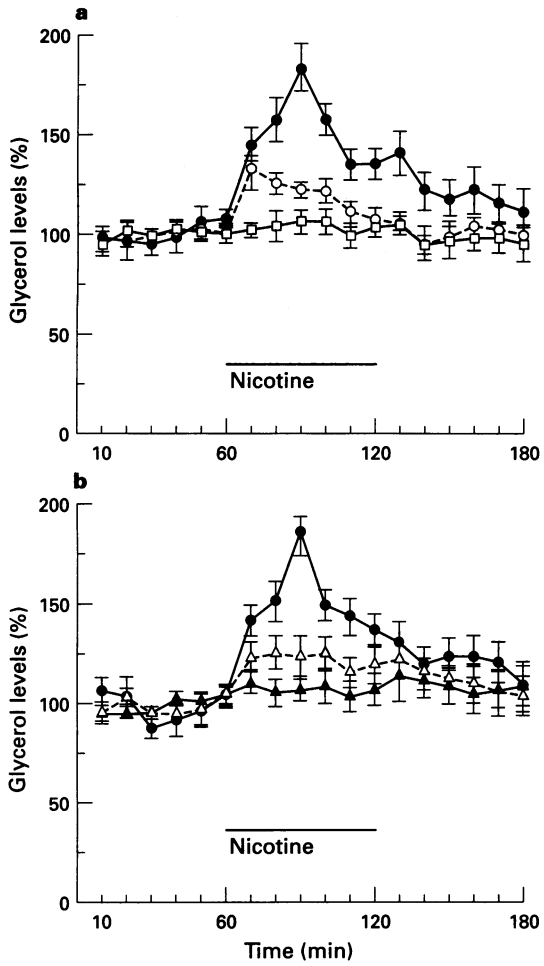

Figure 1 Effects of nicotine on changes in glycerol levels (means ± s.e.) in adipose tissue. Microdialysis was performed at rest for 3.5 h using the standard ethanol-Ringer dialysis solvent. The first 30 min were used only to determine a baseline glycerol level. The remaining 3 h period is depicted and was used for statistical evaluation: 1 h pretreatment, 1 h treatment, 1 h post-treatment. Nicotine was given via the microdialysis probe during the second h of the 3 h experimental period. (a) Effects of a 1 h nicotine infusion: the following nicotine concentrations were used 0.003 μM ($n=6$, □—□) 30 μM ($n=9$, ○- - -○) and 1 mM ($n=10$, ●—●). Values are given as a percentage of the mean glycerol levels during the first 0.5 h baseline period. The baseline glycerol levels were 25 ± 3 μM, 23 ± 3 μM and 24 ± 4 μM, respectively. These values did not differ statistically. The changes in glycerol levels during the whole time course (3 h) for the three nicotine experiments differed statistically when compared with each other ($P<0.01$ by ANOVA). *Post-hoc* analysis revealed that both the time effects and the concentration effects were significant only during the 1 h treatment period with nicotine. (b) Shows the results, using standard microdialysis solvent supplemented with or without mecamylamine (Mec), on the effects of a 1 h nicotine (1 mM) infusion. These experiments were otherwise constructed, presented and statistically evaluated as the experiments in (a). Mecamylamine, which was present during the whole experiment, was added in the following concentrations: 0 ($n=8$, ●—●) 10 μM ($n=6$, △- - -△) and 1 mM ($n=8$, ▲—▲). The mean glycerol levels during the first h of microdialysis were 26 ± 3 μM, 26 ± 2 μM and 27 ± 4 μM, respectively. These values did not differ statistically. The whole time course (3 h) for the three types of experiments differed in a significant way when compared with each other ($P<0.01$ by ANOVA). *Post-hoc* analysis revealed that both the time effects and the concentration effects were significant only during the 1 h treatment period with nicotine. The line indicates when nicotine was given.

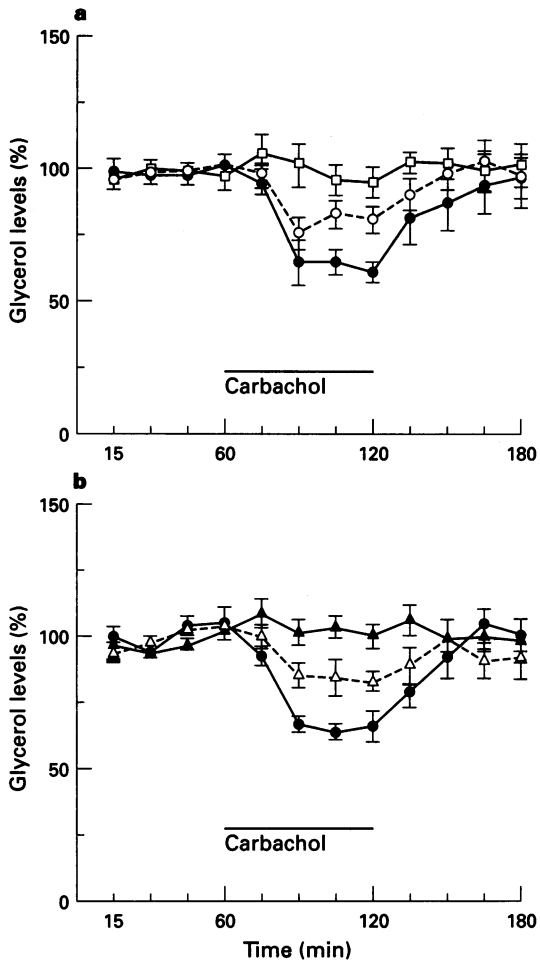

Figure 2 Effects of carbachol (mean ± s.e.) on changes in dialysate glycerol levels. (a) The effects of carbachol (given during second h) using the standard dialysis solvent. The following carbachol concentrations were used: 1 μM ($n=6$, □—□) 10 μM ($n=10$, ○- - -○) and 1 mM ($n=6$, ●—●). The baseline values for glycerol were 29 ± 3 μM, 28 ± 4 μM and 32 ± 5 μM, respectively. These values did not differ statistically. When changes in glycerol levels during the whole time course (3 h) for the three experiments were tested, a significant difference was observed between the curves ($P<0.01$ by ANOVA). *Post-hoc* analysis revealed that the effects of time and concentration were statistically significant during the time when carbachol was given (i.e. 1 h treatment period). (b) The effect of a 10 μM dose of carbachol given during the second h when the dialysis solvent was supplemented with atropine, 0 ($n=8$, ●—●); 0.1 μM ($n=6$, △- - -△); 10 μM ($n=9$, ▲—▲) during the whole experiment. The baseline values for glycerol levels did not differ statistically between the groups: 0 atropine: 24 ± 4 μM, 0.1 μM atropine: 25 ± 5 μM and 10 μM atropine: 28 ± 3 μM. The changes in the glycerol levels of the whole time course (3 h) in the different experiments differed statistically between each other ($P<0.05$ by ANOVA). *Post-hoc* analysis revealed that the effects of time and concentration were statistically significant during the time when carbachol was given (i.e. 1 h treatment period). Further details explained in the legend to Figure 1.

over time in the ethanol ratio during the 3 h experimental period (graph not shown). Thus, neither mecamylamine nor atropine itself had an effect on the local blood flow.

The action *in vivo* of the endogenous neurotransmitter, acetylcholine, was also investigated (Figure 4). These effects were studied only during muscarinic or nicotinic receptor blockade, because of the putative dual effect of acetylcholine on glycerol levels. A net increasing or decreasing effect by acetylcholine alone is dependent on the balance between the two receptor classes. When the adipose tissue was pretreated with atropine (0.01 mM for 1 h), the addition of acetylcholine (1 mM) for 1 h rapidly increased ($P<0.01$) the dialysate glycerol levels, which returned to the baseline levels during the third hour. The peak effect was about 35% above the baseline value. When the tissue was pre-exposed to mecamylamine (0.01 mM), the subsequent addition of 1 mM acetylcholine caused a significant ($P<0.001$) and sustained reduction in dialysate glycerol. The nadir effect was a 50% reduction, as compared to the baseline value. When acetylcholine was removed from the medium in the mecamylamine experiment, the glycerol levels rapidly returned to baseline levels. The effects of acetylcholine plus mecamylamine, as compared to acetylcholine plus atropine, were significantly different ($P<0.001$ by ANOVA). Acetylcholine in the presence of atropine or mecamylamine had no effect on the ethanol ratio.

The *in situ* effects of cholinoceptor agonists were also studied under conditions when there was minimal or no influence of the adrenergic system (Figure 5). Adipose tissue was microdialyzed with 0.1 mM propranolol (non-selective β-adrenoceptor blocker) plus 0.1 mM phentolamine (non-selective α-adrenoceptor blocker), which was present from the beginning of the study. Nicotine or carbachol was added (1 mM) during the last 2 h. This deviation from the original protocol was used because the onset of nicotine's action was retarded under complete α- plus β-adrenoceptor blockade. The addition of nicotine caused a gradual increase in the dialysate glycerol levels, which reached a level that was 50% above the baseline at the end of the experiment. The addition of carbacholine caused a rapid, sustained decrease in the glycerol levels. The maximum effect (at nadir) was a 35% decrease, as compared to the baseline glycerol levels. The effects of nicotine and carbachol differed significantly when compared with each other ($P<0.01$ by ANOVA). Neither nicotine nor carbachol influenced the ethanol ratio, which varied between 65% and 75% throughout the experimental period. The ethanol ratio in the nicotine and carbachol experiments did not differ statistically

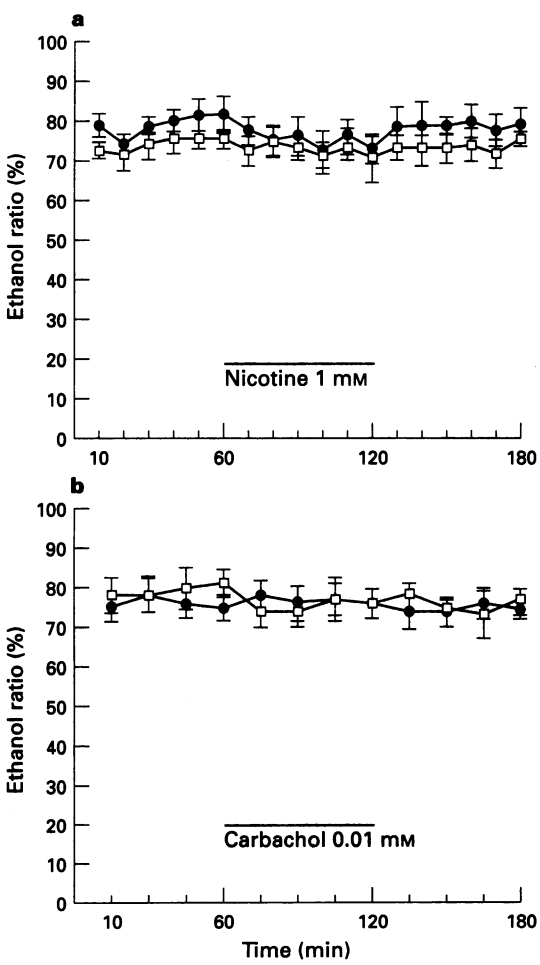

Figure 3 Effects of nicotine (1 mM, a) or carbachol (10 μM, h) on the ethanol ratio (means ± s.e.) in the absence (●) or presence (□) of mecamylamine (Mec) (0.1 mM) or atropine (10 μM). There was no significant difference between the groups of values in (a) and (b), testing the dose versus time with ANOVA ($P<0.4$). These experiments were the same as presented in Figure 1 and 2 but ethanol was measured instead of glycerol.

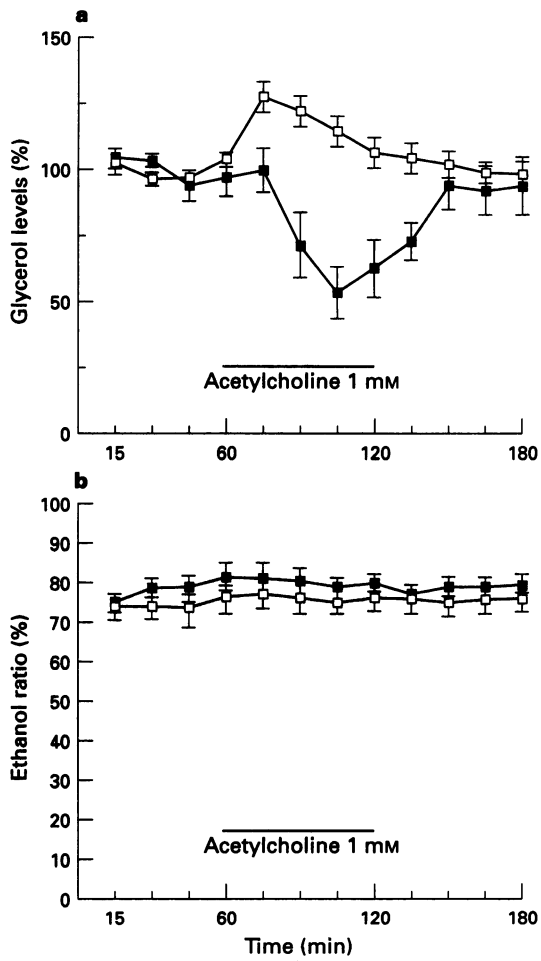

Figure 4 Effects (means ± s.e.) of acetylcholine (1 mM) on changes in the glycerol levels (a) and the ethanol ratio (b). The dialysis solvent was supplemented with either mecamylamine (Mec) (10 μM, ■) or atropine (10 μM, □). Further details are given in the legend to Figure 1. There was no difference in the baseline glycerol levels in the two groups (data not shown). The mecamylamine and atropine experiments were also compared statistically as regards the whole time course with ANOVA. No differences were observed in the ethanol experiments ($P=0.28$). However, the changes in glycerol levels differed significantly ($P<0.001$ by ANOVA). The effects of time and drug were statistically significant during the 1 h treatment period as judged by *post-hoc* analysis.

when compared with each other ($P=0.31$ by ANOVA). Methodological experiments revealed that the combination of propranolol and phentolamine described above did not change the baseline glycerol levels or ethanol ratios.

As described in the legends to the figures there was a small deviation in baseline glycerol levels between the different experiments. However, this variation was not statistically significant when all experiments were compared, as measured by ANOVA. In addition, there was no relationship between basal glycerol levels, on the one hand, and changes in the glycerol levels induced by nicotine, carbacholine or acetylcholine, on the other hand, as judged from statistical evaluation using linear regression analysis.

ANOVA and *post-hoc* analysis of the whole time course was used to evaluate statistically the concentration-dependency of added drugs. Similar results were obtained if the area under the curve during the 1 h treatment period was used for statistical comparison (values not shown).

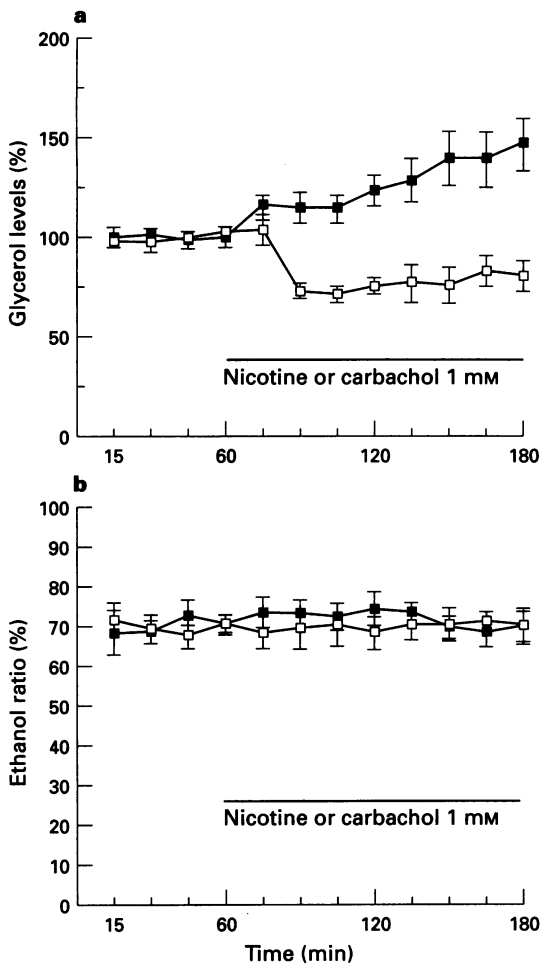

Figure 5 Effects (means ± s.e.) of 1 mM of nicotine or carbachol on glycerol levels (a) or ethanol ratio (b) during total adrenoceptor blockade. The standard dialysis solvent also contained 0.1 mM propranolol and 0.1 mM phentolamine from the beginning of the experiment. Nicotine (1 mM, ■) or carbachol (1 mM, □) was added during the last 2 h of experiment. Further details are given in the legend to Figure 1. As regards the absolute baseline glycerol values, there was no significant difference between the nicotine and carbachol experiments (data not shown). The whole time course for the two types of experiments was compared by ANOVA. The changes in glycerol levels differed significantly when the experiments with nicotine and carbachol were compared with each other ($P<0.001$) by ANOVA. *Post-hoc* analysis revealed the effects of drug and time were statistically significant during the last 2 h of the experiment. No such difference was observed in ethanol ratios between these experiments ($P=0.46$).

The *in vitro* effects of the cholinoceptor agonists on glycerol release were investigated in isolated fat cells. In one set of experiments ($n=4$) fat cells were incubated with increasing concentrations of nicotine (0, 1 μM, 10 μM, 100 μM, 1 mM). The corresponding glycerol values (μmol g^{-1} lipid 2 h^{-1}) were 2.0 ± 0.1, 2.0 ± 0.2, 2.4 ± 0.3, 2.0 ± 0.2 and 2.0 ± 0.3. These values did not differ statistically. In another set of experiments ($n=4$) the cells were incubated with carbachol (0.1 μM, 10 μM, 100 μM). The corresponding glycerol values, which did not differ statistically, were 1.8 ± 0.2, 1.9 ± 0.3, 1.9 ± 0.2 and 1.8 ± 0.2. Lipolysis interactions between noradrenaline, nicotine and carbachol were also investigated in fat cells ($n=4$). Noradrenaline caused a dose-dependent stimulation of glycerol release with a half-maximum effect at 10 ± 2 nmol l^{-1}; the maximum effect was 310 ± 30% stimulation of the baseline value. Neither nicotine (0.01, 0.1 or 1 mM) nor carbachol (0.1 or 1 mM) treatment had any effect on noradrenaline-induced glycerol release (graph not shown).

Discussion

This study demonstrates, for the first time, the presence of functional cholinoceptors in adipose tissue *in vivo*. Activation of nicotinic cholinoceptors stimulates lipolysis, whereas activation of muscarinic cholinoceptors inhibits lipolysis in human adipose tissue. These effects appear, first, to be independent of the adrenergic system, and, secondly, not to be influenced by changes in the local blood flow in adipose tissue.

The microdialysis method measures the concentration of glycerol in the extracellular space of adipose tissue. As reviewed, changes in these glycerol levels during kinetic experiments reflect production/reutilization by fat cells and removal of adipose tissue glycerol by venous blood flow (Arner & Bülow, 1993). There is no inflow of arterial glycerol, since the glycerol levels in the interstitial space of adipose tissue are 2–3 times higher than the circulating levels (Arner & Bülow, 1993). Reutilization of glycerol in the adipose tissue takes place only to an insignficant extent, and cannot contribute to the changes in glycerol levels, following nicotine treatment.

In the study, glycerol was used as an index of lipolysis (i.e., hydrolysis of triglycerides in fat cells). Unfortunately, it is not possible to determine the source of glycerol in microdialysis experiments. In theory, glycerol could originate from other intracellular sources, such as phospholipids. However, these lipids constitute less than 1% of the water-free mass of fat cells, whereas the triglycerides constitute over 95% of this mass. Extracellular triglycerides in lipoproteins may also be a source of glycerol, that can be liberated from lipoproteins through the action of lipoprotein lipase. However, activation of lipoprotein lipase *in vivo* is a slow process, as reviewed by Alihaud (1990). In the present study, we obtained effects (which were often maximal) within 30 min. Futhermore, recent studies using arteriovenous measurements have suggested that less than 10% of glycerol, derived from human abdominal subcutaneous adipose tissue originates from sources outside the fat cells (Frayn, 1992; Kurpad *et al.*, 1994).

In adipose tissue and skeletal muscle the ethanol ratio has been used to study local blood flow around the microdialysis probe (Hickner *et al.*, 1991; Galitsky *et al.*, 1993). A thorough methodological evaluation of this technique was made *in vitro* and *in vivo* in skeletal muscle, including a direct comparison with the radioactive xenon-washout technique (Hickner *et al.*, 1992; 1994). These data suggest that changes in the ethanol ratio reflect changes in the flow around the probe *in vivo*. There is no reason to believe that adipose tissue should differ from muscle in this respect. However, there is no alternative to the ethanol technique for the present type of experiments, where local blood flow must be measured at the place where the microdialysis probe is situated. This rules out the use of a laser doppler probe, which has to be placed distant from the microdialysis probe. The xenon-washout method is also not suitable, since xenon must be injected directly into the tissue.

Microspheres cannot be used to evaluate blood flow in man for ethical reasons. In a previous microdialysis study, we evaluated the ability of the ethanol technique to detect changes in local blood flow of human adipose tissue, using vasodilators (nitroprusside or hydralazine) and a vasoconstrictor agent (clonidine), which were added to the dialysis solvent (Galitsky *et al.*, 1993). These drugs caused changes in the ethanol escape which were in harmony with their expected effects on blood flow. It is not likely that the ethanol technique is too insensitive to measure changes in blood flow. Small effects were detected *in situ* using selective phosphodiesterase inhibitors in human adipose tissue (Arner *et al.*, 1993). A clear concentration-dependency following *in situ* stimulation with a β-adrenoceptor agonist was also observed in the same tissue (Enoksson *et al.*, 1995). Finally, the ethanol technique can detect small physiological increases (30–50%) in blood flow rate in human skeletal muscle, which occurs during mental stress (Hickner, 1995).

Neither nicotine nor carbachol changed the ethanol ratio, indicating that they did not change local adipose tissue blood flow. Thus, the observed changes in glycerol levels most probably reflect the production of glycerol through stimulation or inhibition of lipolysis in the fat cells although a minor effect due to minimal (and undetected) changes in blood flow cannot be excluded. To support this notion further measurements of fatty acids (the other end-product of lipolysis) would have been of importance, but due to their hydrophobic nature it was not possible to monitor fatty acids in the extracellular space of adipose tissue by microdialysis.

Nicotine administration *in situ* rapidly stimulated adipose tissue lipolysis in a concentration-dependent fashion, although rather high concentrations had to be used in order to get marked effects. However, a small but statistically significant effect was obtained with as little as 0.3 μM nicotine. At rest, a 100% increase in the glycerol levels in adipose tissue was found, using the highest nicotine concentration (1 mM) in the dialysis solvent. It should be borne in mind, that the actual concentration of nicotine (and of the other drugs used in this study) is much lower at the target cell level. *In vivo* recovery in the present experimental situation is about 10% (Arner *et al.*, 1990), meaning that the concentration of drug leaving the probe is 10 times lower than the concentration entering. Thereafter, a further but unknown dilution of the drug occurs when it is distributed in the interstitial space. Methodological data (Beneviste *et al.*, 1989) and theoretical predictions (Bungay *et al.*, 1990) suggest that the concentration of a drug leaving the probe rapidly decreases in the area 1 mm from the probe.

The specificity of the nicotine effect was tested with the antagonist, mecamylamine. It would be ideal to use highly selective competitive antagonists such as trimethapan, dihydrobeta-erythroidine or methyllyconitine. For ethical reasons, however, it was not possible to use these agents in human experiments. Ethical approval was obtained only for the use of mecamylamine or hexamethonium, which have been given to man previously and found to be safe. Hexamethonium was avoided because it is a channel blocker (Grenhoff & Svensson, 1989) and does not pass tissue barriers, such as the blood brain barrier (Greenwood *et al.*, 1992). Mecamylamine, on the other hand, crosses tissue barriers and is generally classified as a nicotinic receptor blocker, although it can act as a channel blocker in lobster skeletal muscle (Grenhoff & Svensson, 1989). Although mecamylamine in some tissues is a non-competitive nicotine antagonist, it is frequently used in animal studies in order to test the specificity of nicotine-induced changes. For example, we have observed that mecamylamine pretreatment counteracts nicotine-induced changes in catecholamine nerve terminal networks in the brain, and in neuroendocrine function (Andersson *et al.*, 1984; Andersson, 1985). In the present study, the lipolytic action by nicotine was blocked in a concentration-dependent way by pretreatment with mecamylamine. The nicotine effect could be almost completely (i.e., 90%) blocked by an equimolar concentration of mecamylamine. In addition, the nicotine effect on lipolysis was also observed, although it was somewhat delayed, during complete α- plus β-adrenoceptor blockade with phentolamine plus propranolol. Taken together, these results indicate that lipolysis is stimulated via a nicotinic cholinoceptor acting separately from the classical α- and β-adrenoceptor-mediated effects on lipolysis.

It is well established that lipolysis in adipose tissue is stimulated by several hormones, which bind to receptors that are coupled to adenylyl cyclase via the G_S protein (Hirsch *et al.* 1989). A direct role of the cholinoceptors in regulating lipolysis or other metabolic events has not previously been demonstrated. However, the present study clearly shows that lipolysis was stimulated through a nicotinic cholinoceptor mechanism. Nicotinic cholinoceptors function independently of G-proteins and adenylyl cyclase (Changeux *et al.*, 1987). Thus, the data demonstrate a new way of metabolic regulation *in vivo*, namely, stimulation of lipolysis by a receptor which acts through a chemically gated ion channel.

A number of selective muscarine-like agonists are available for studies *in vitro* or *in vivo* in laboratory animals. For the ethical reasons discussed above, we were allowed to use only carbachol or pilocarpine in human microdialysis experiments *in vivo*. Carbachol was preferred because it is a full agonist with high muscarine receptor affinity, whereas pilocarpine is a partial agonist with low affinity for muscarine receptors (Freedman *et al.*, 1988). Although carbachol may have some nicotine-like effects, it was recently classified as a muscarine receptor agonist (Niroomand *et al.*, 1992). The administration of carbachol rapidly inhibited lipolysis in adipose tissue in a concentration-dependent fashion. This antilipolytic effect was muscarine receptor-specific, since it could be completely counteracted in a dose-dependent fashion by the highly selective muscarinic receptor antagonist, atropine. Furthermore, a clear antilipolytic effect of carbachol was observed under complete α/β-adrenoceptor blockade with phentolamine plus propranolol. These findings indicate, that the muscarine-like cholinoceptor, like the nicotinic cholinoceptor, acts on lipolysis independently of the adrenoceptor systems.

Muscarinic receptors are coupled to G-proteins and exert many of their effects through adenylyl cyclase (Housaly, 1992). As discussed, slight glycogen synthesis in the liver can be demonstrated following parasympathetic stimulation (Guyton, 1986). This effect may be mediated by muscarinic receptors. So far as we know, however, the present data provided the first evidence, that marked metabolic effects are specifically mediated by muscarine-like cholinoceptors.

The findings with the endogenous neurotransmitters, acetylcholine, further support a physiological role of cholinoceptors in adipose tissue, Thus, acetylcholine decreased glycerol levels during nicotine receptor blockade by mecamylamine, and increased glycerol levels during muscarine receptor blockade by atropine, but it had no effect on local blood flow. We performed no detailed examination of the action of acetylcholine, since the neurotransmitter is rapidly metabolized in peripheral tissues (Guyton, 1986; Lefkowitz *et al.*, 1990; McIsaac, 1992). The conditions for maximum inhibition of acetylcholine breakdown in microdialysis experiments are not yet worked out. In addition, the action of acetylcholine alone will depend on the balance between nicotinic and muscarinic receptors, which is unknown. In order to sort this out it is necessary to peform extensive experiments with various doses of acetylcholine and the selective antagonists simultaneously. In addition, it is necessary to work out optimal conditions to inhibit the marked breakdown of acetylcholine that occurs *in vivo*. This may not be possible for ethical reasons, since the powerful inhibitors of acetylcholine breakdown have not been proved safe for human use. Furthermore, the physiological action of acetylcholine in human adipose tissue was beyond the scope of this study, which focused on receptor classification rather than on acetylcholine action.

The mechanisms by which cholinoceptors regulate lipolysis remain to be established. It is possible that the receptors are

functionally expressed in fat cells. This is unlikely, since no effect of nicotine or carbachol on glycerol release was observed in human subcutaneous fat cells incubated *in vitro*. In theory, the receptors on fat cells might have lost functional activity during *in vitro* preparation. If so, they are studied best *in situ* with, for example, microdialysis. Alternatively, the findings with nicotine and carbacholine may be related to receptors, which are situated on cells other than adipocytes in adipose tissue, releasing lipolytic and/or antilipolytic transmitters, acting directly or indirectly on the fat cells. Release of paracrine lipolytic agents originating from endothelial cells of capillaries, and interacting with circulating lipids must also be considered to explain the effects of the compounds. Further studies are needed to answer these questions. Indirect evidence of a functional nicotine receptor have recently been provided in pieces of human adipose tissue in organ culture (Chajek-Shaul *et al.*, 1994).

Another topic to be discussed is the physiological relevance of the present findings, since the evidence for a cholinergic innervation of adipose tissue is limited as discussed previously (Rosell & Belfrag, 1979; Fredholm, 1985). Acetylcholine and noradrenaline are considered as the main transmitters of the postganglionic parasympathetic and sympathetic neurones, respectively. On one hand, no parasympathetic innervation has so far been demonstrated in the adipose tissue. On the other hand, sympathetic postganglionic neurones innervating the piloerector muscle of the skin train, the sweat glands, as well as skin blood vessels (which also receive a noradrenergic innervation) use acetylcholine as transmitter.

It is suggested that as much as 20% of postganglionic sympathetic neurones could be cholinergic (Lindh & Hökfelt, 1990). Thus, acetylcholine could be released in the vicinity of the adipose call and directly or indirectly (as discussed above) regulate lipolysis. From this discussion it still may be argued that cholinoceptors in adipose tissue are dormant receptors with little physiological role. Even so, they could play a pathophysiological role, and, in particular, the findings of nicotine-induced lipolysis may have clinical implications. It is well known that tobacco smoking affects body weight regulation and lipoprotein metabolism. A relationship between nicotine use and atherosclerotic cardiovascular disease is firmly established, but the mechanisms responsible for these side-effects of smoking are largely unknown, as discussed (McBride, 1992). There is a well-documented effect of nicotine on sympathetic ganglia following systemic delivery, which involves a nicotine-induced activation of the postganglionic noradrenergic sympathetic neurones, which *inter alia* innervate the adipose tissue. In addition, the present results suggest, that a direct lipolytic effect of nicotine through its own receptor in the adipose tissue can contribute to the disturbances in lipid metabolism in smokers.

Whether the nicotinic and muscarinic receptors are metabolically functional only in human adipose tissue, or are found in other species and human tissues as well, remains to be established. The nicotinic and muscarinic cholinoceptors constitute two large receptor families, in which the different subtypes may have specific effects, as reviewed (Deneris *et al.*, 1991; Brann *et al.*, 1992). Any of these receptor subtypes may be involved in the process of lipolysis.

In summary, this study demonstrates the existence of functional nicotinic and muscarinic cholinoceptors with opposite effects on lipolysis *in vivo*, in man. Further studies are necessary in order to understand the physiological role and molecular events that are involved in the cholinoceptor-mediated effects on lipolysis.

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