CLINICAL STUDY

Role of insulin as a negative regulator of plasma endocannabinoid levels in obese and nonobese subjects

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Abstract

Objective: Endocannabinoids (ECs) control metabolism via cannabinoid receptors type 1 (CB₁). Their plasma levels are elevated in overweight type 2 diabetes (T2D) and in obese patients, and decrease postprandially in normoweight individuals. We investigated in two different cohorts of nonobese or obese volunteers whether oral glucose in glucose tolerance tests (OGTT) or acute insulin infusion during euglycemic hyperinsulinemic clamp affect plasma EC levels.

Design and methods: OGTT was performed in ten obese hyperinsulinemic patients (body mass index (BMI) = 35.8 kg/m², fasting insulin = 14.83 mU/l), and ten normoweight normoinsulinemic volunteers (BMI = 21.9 kg/m², fasting insulin = 7.2 mU/l). Insulin clamp was performed in 19 mostly nonobese men (BMI = 25.8 kg/m²) with varying degrees of liver fat and plasma triglycerides (TGs), with (n = 7) or without T2D. Plasma levels of ECs (anandamide and 2-arachidonoylglycerol (2-AG)) were measured by liquid chromatography–mass spectrometry, before and 60 and 180 min after OGTT, and before and 240 and 480 min after insulin or saline infusion.

Results: Oral glucose load decreased anandamide plasma levels to an extent inversely correlated with BMI, waist circumference, subcutaneous fat, fasting insulin and total glucose, and insulin areas under the curve during the OGTT, and nonsignificantly in obese volunteers. Insulin infusion decreased anandamide levels to an extent that weakly, but significantly, correlated negatively with TGs, liver fat and fasting insulin, and positively with high density lipoprotein cholesterol. OGTT decreased 2-AG levels to a lower extent and in a way weakly inversely correlated with fasting insulin.

Conclusions: We suggest that insulin reduces EC levels in a way inversely related to anthropometric and metabolic predictors of insulin resistance and dyslipidemia.

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Introduction

The endocannabinoid (EC) system and, in particular, the cannabinoid receptor type 1 (CB₁) and its endogenous agonists, the ECs anandamide and 2-arachidonoylglycerol (2-AG), are emerging as major players in the control of metabolism and, when malfunctioning, as one of the underlying causes of obesity, hyperglycemia, dyslipidemia, ectopic fat, insulin resistance, and type 2 diabetes (T2D) (1, 2). Animal studies have shown that antagonism of EC action at CB₁ receptors counteracts body weight gain as well as the metabolic consequences of high fat diets in both mice and rats (3–5), whereas experimental EC overactivation causes CB₁-mediated elevation of triglycerides (TGs) in lean mice (6). Clinical studies carried out in obese patients with the CB₁ receptor antagonists, rimonabant

and taranabant, have shown that these compounds cause significant weight loss and metabolic benefits in terms of reduction in insulin resistance and high TGs, and increase in high density lipoprotein (HDL) cholesterol (7, 8). Several human studies have found strong associations between high plasmatic EC levels and intra-abdominal obesity, high TGs, low HDL cholesterol, and insulin resistance in obese (9–11) as well as T2D patients (12). Since the concentrations of both anandamide and particularly 2-AG in the human plasma are, at best, tenfold lower than in most tissues, and in view of the fact that ECs are not circulating hormones but rather local mediators (1), it has been postulated that these measures reflect ‘spill-over’ of these compounds from peripheral tissues, rather than biologically active concentrations in blood cells. Accordingly, dysregulation of the EC levels is also observed in animal models of

obesity, where it consists of an early and/or persistent elevation of anandamide and/or 2-AG levels in the liver, epididymal fat, brown fat, pancreas, and skeletal muscle and of their reduction in the subcutaneous adipose tissue (4, 12–15). Such alterations have been proposed to result in CB₁-mediated accumulation of fat in nonsubcutaneous depots and liver, reduction in adiponectin production, and insulin resistance in the liver and skeletal muscle, associated with reduced energy expenditure and enhanced insulin secretion from the pancreas (1).

Among the possible causes of the peripheral dysregulation of the EC system, the lack of negative control by insulin on EC levels has been suggested by previous studies (12, 16, 17). In particular, experiments in isolated cells have shown that conditions mimicking high glucose and/or insulin resistance can enhance EC levels in models of adipocytes and pancreatic β -cells (12, 17). In contrast, anandamide levels are reduced following incubation of 'normoglycemic' β -cells with insulin, or in the postprandial plasma of normoglycemic normoweight patients (12). Accordingly, the expression of the anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH), is elevated in insulin-treated subcutaneous adipocytes from normoglycemic nonobese subjects (16). However, no direct evidence exists for a cause–effect relationship between insulin action and decreased EC levels *in vivo* in humans, and it is not known how metabolic factors such as obesity and hyperglycemia might influence this relationship.

In the present study, we investigated in two different cohorts of nonobese and obese volunteers whether the insulin response to oral glucose load in oral glucose tolerance tests (OGTT) or to acute insulin infusion during euglycemic hyperinsulinemic clamp (EHC) affects plasma EC levels, which have been used here and previously (9–11, 18, 19) as an indirect measure of peripheral EC system activity. Furthermore, we have studied the potential impact of various anthropometric and metabolic parameters on the putative changes in EC levels induced by these interventions.

Materials, subjects, and methods

Subjects

Two different cohorts of volunteers were used for the two studies carried out here. In the first study, in which the effect of an oral glucose load on EC levels was investigated, ten normoweight and ten obese volunteers, under no medication that could affect glucose or insulin levels, were employed (Table 1). In the second study, in which the effect of insulin infusion on EC levels was investigated, 19 volunteers within a wide range of body mass index (BMI) participated (Table 2). Seven of these patients had T2D, two of whom were treated with diet alone, two with diet and sulfonylurea, and three

Table 1 Metabolic and anthropometric parameters of the normoweight and obese volunteers participating in the oral glucose tolerance test (OGTT) experiments. Values were compared by using the Mann–Whitney test.

	Normoweight (n=10)	Obese (n=10)	Statistical significance
Gender	6F/4M	6F/4M	
Age (years)	44 $\pm$ 2	44 $\pm$ 2	<i>P</i> >0.1
Weight (kg)	68.1 $\pm$ 4.0	108.4 $\pm$ 4.7	<i>P</i> =0.0002
BMI (kg/m ²)	21.9 $\pm$ 0.6	35.8 $\pm$ 1.1	<i>P</i> =0.00016
Waist (cm)	78.0 $\pm$ 2.2	114.0 $\pm$ 2.6	<i>P</i> =0.00016
L4–L5 visceral fat (cm ²)	63.8 $\pm$ 8.7	187.2 $\pm$ 15.6	<i>P</i> =0.00021
L4–L5 subcuta- neous fat (cm ²)	180.6 $\pm$ 24.8	537.0 $\pm$ 47.5	<i>P</i> =0.00021
Fasting glucose (mmol/l)	4.58 $\pm$ 0.12	4.97 $\pm$ 0.16	<i>P</i> =0.0887
Fasting insulin (mU/l)	7.2 $\pm$ 1.0	14.8 $\pm$ 1.7	<i>P</i> =0.00051
Triacylglycerols (mmol/l)	0.89 $\pm$ 0.054	2.45 $\pm$ 0.33	<i>P</i> =0.00045
AUC glucose	18 971 $\pm$ 960	21 785 $\pm$ 921	<i>P</i> =0.049
AUC insulin	4839 $\pm$ 446	13 334 $\pm$ 2385	<i>P</i> =0.0032

with a combination therapy of sulfonylurea, metformin, and diet. The other subjects were taking no medication that could affect glucose or insulin levels. All subjects gave informed consent to the study and received appropriate treatments. The study design was approved by the ethics committees of Helsinki University Central Hospital and of the Antwerp University Hospital, and each participant gave written informed consent. All samples were collected in accordance with the Declaration of Helsinki.

Methods for the assessment of anthropometric and metabolic parameters

All anthropometric measurements were performed in the morning, with patients in fasting conditions and undressed. BMI was calculated as weight in kilograms over height² in square meters. Waist circumference was measured at the mid-level between the lower rib margin and the iliac crest. Body composition was determined by bioimpedance analysis as described by Lukaski *et al.* (20), and fat mass percentage was calculated using the formula of Deurenberg *et al.* (21). In the normoweight and obese volunteers participating in the first study, a computer tomography-scan at L4–L5 level was performed to measure the cross-sectional area of total abdominal adipose tissue area, visceral abdominal adipose tissue (VAT), and subcutaneous abdominal adipose tissue (SAT) according to previously described methods (22). First, the total area of abdominal adipose tissue was measured at –190 to –30 Hounsfield units. Subsequently, the area of VAT was distinguished from SAT by manually tracing the abdominal muscular wall separating the two adipose tissue compartments. In these patients, a fasting blood sample was taken

Table 2 Metabolic and anthropometric parameters of the normo-weight/overweight borderline male volunteers participating in the euglycemic hyperinsulinemic clamp (EHC) experiments. Values were compared by using the Mann–Whitney test.

	Nondiabetic (n=12)	With type 2 diabetes (n=7)	Statistical significance
Gender	12M	7M	
Age (years)	49.5±3.0	56.3±3.8	P>0.1
Weight (kg)	82.2±2.6	90.9±3.3	P=0.047
BMI (kg/m ²)	26.3±0.8	28.5±1.6	P>0.1
Waist (cm)	95.4±2.5	101.4±2.5	P>0.1
L4–L5 visceral fat (cm ²)	121.2±14.3	148.7±24.2	P=0.022
L4–L5 subcutaneous fat (cm ²)	164.5±13.4	179.0±17.8	P>0.1
Liver fat (%)	2.8±0.7	11.7±2.5	P=0.00295
ALAT (unit/l)	27.5±2.0	37.1±6.1	P>0.1
Fasting glucose (mmol/l)	5.7±0.2	9.3±1.2	P=0.0006
Fasting insulin (mU/l)	5.5±1.0	12.9±2.5	P=0.006
Triacylglycerols (mmol/l)	1.32±0.12	2.57±0.30	P=0.003
M value (μmol/kg per min)	7.22±0.53	4.49±1.14	P=0.028
ApoB (mg/dl)	106.3±6.7	125.6±4.2	P=0.063
HDL cholesterol (mmol/l)	1.38±0.49	1.01±0.53	P=0.0013
Adiponectin (ng/ml)	9606.8± 1059.5	6831.4± 1038.3	P>0.1

from an antecubital vein to determine the fasting levels of TGs, glucose, and insulin. An OGTT was performed with 75 g of glucose, with blood samples taken to determine glucose in the fasted state and 15, 30, 60, 90, 120, 150, and 180 min after the glucose load. During the OGTT, insulin was also determined 30, 60, 120, and 180 min after the glucose load. Plasma glucose and TGs were measured on Vitros 750 XRC (Ortho Clinical Diagnostics, Johnson & Johnson, High Wycombe Bucks, UK). Insulin levels were measured with the Medgenic two-site IRMA assay (BioSource, Nivelles, Belgium). The total glucose and insulin areas under the curve (AUC) during the OGTT were determined with the trapezoid method (23).

In the cohort of volunteers employed in the second study, subcutaneous and intra-abdominal fat volumes and liver fat content were measured using magnetic resonance imaging and magnetic spectroscopy as previously described (24). The mean of 16 T-weighted transaxial scans at L4–L5 level was used to calculate the intra-abdominal and subcutaneous fat areas (cm²). The biochemical analyses of plasma TGs, ApoB, HDL cholesterol, insulin, and glucose were carried out as previously described (25), and alanine aminotransferase (ALAT) was determined according to European committee for Clinical Laboratory Standards. Fasting plasma adiponectin levels were determined with the human adiponectin ELISA kit (B-Bridge International, San Jose, CA, USA).

Differences in the anthropometric and metabolic parameters between nonobese and obese subjects in the first study, and between nondiabetic and T2D subjects in the second study, were evaluated by means of the Mann–Whitney *U* test.

EHC studies The patients were admitted at 0730 h after an overnight fast. An indwelling cannula was inserted into an antecubital vein for infusions, and a second cannula was inserted retrogradely into a heated hand vein to obtain arterialized venous blood for sampling. At 0 min, a primed-continuous infusion of insulin (Human Actrapid; Novo Nordisk, Copenhagen, Denmark) was started at a rate of continuous infusion of 1 mU/min per kg. Plasma glucose was maintained at its fasting level by a variable rate infusion of glucose (200 g/l) (26). The infusion rate was determined empirically based on plasma glucose measurements that were performed every 5–10 min from arterialized venous blood. Whole body insulin sensitivity (*M* value) was calculated from the mean values of the second hour of glucose infusion rate after correcting for changes in the glucose pool size (26) and was expressed as μmol/min per kg body weight. Insulin infusion continued for 8.5 h, and the participants remained fasting until 1700 h when they were served a hospital meal. During the control study, saline was infused at a rate of 200 ml/h, which is approximately equal to volume of insulin and glucose infusions during the clamp. The blood samples for measurements of plasma EC levels were taken 30 min before insulin/saline infusions and at 240 and 480 min following the initiation of insulin/saline infusions.

Measurement of plasmatic EC levels

Blood samples were taken on heparin, immediately centrifuged free of erythrocytes at 4 °C, and then kept frozen at –80 °C until analysis. Under these conditions, ECs are stable towards oxidation for up to 6 months. Within 6 months of blood sampling, EC levels were measured by isotope dilution liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) as previously described (10). In particular, before thawing, d₅-2-AG (5 pmol) and d₈-anandamide (5 pmol), obtained from Cayman Chemicals (Ann Arbor, MI, USA), were added to each sample, and lipids were then extracted and prepurified as described (10). The isomerization of 2-AG to its 1- and 3-isomers occurring during the extraction, purification, and LC–MS analysis (27) was taken into account since also the internal deuterated 2-AG underwent similar isomerization, and the chromatographic procedure used (C18 Phenomenex column, 15 × 1.5 cm, 3 μm particle size, eluted isocratically with methanol/water/acetic acid 85:15:0.1 by vol.) allows for the separation of 2-AG (11 min) from 1(3)-AG (12.5 min). Therefore, the peaks corresponding to all isomers were added up and quantified by isotope dilution with those of the internal deuterated standard.

Data are reported as means $\pm$ S.E.M. Differences in the amounts of ECs following OGTT in nonobese and obese subjects were analyzed by repeated-measures ANOVA. For the insulin clamp study, saline and clamp curves were compared, with correction of the baseline values (before the clamp) using repeated-measures ANCOVA. Differences in the amounts of ECs between two groups of volunteers (i.e. between obese and normoweight, and between normoglycemic and T2D volunteers) were analyzed by the Student–Newman–Keuls's test. Correlations between changes (Δ) in EC levels, normalized to basal levels (Δ/T_0 , $\Delta = T_0 - T$) in the OGTT experiments, or to the corresponding saline infusion levels (Δ/T_{saline} , $\Delta = T_{\text{saline}} - T$) in the EHC experiments, and other variables at baseline were assessed by Pearson's correlations. In the EHC experiments, the EC levels measured after saline infusions at the corresponding times were used as controls since plasma EC levels are known to change during the day and following intervals of time longer than 3 h (V Di Marzo & S Petrosino, unpublished observations). Normalization of Δ values to baseline levels was necessary in the OGTT experiments because in this case the basal plasma levels of anandamide (T_0) were significantly higher in obese than in normoweight volunteers. A P value <0.05 indicated statistical significance.

Results and discussion

Effect of glucose on EC levels in normoweight and obese volunteers

In normoweight/normoinsulinemic volunteers (Table 1), the oral glucose load caused a statistically significant overall reduction in anandamide levels ($P=0.013$, by repeated-measures ANOVA), a reduction at 60 min ($P=0.046$), and a further reduction at 180 min ($P=0.008$; Fig. 1A). Oral glucose load caused a statistically significant overall reduction in 2-AG levels ($P=0.028$), and a reduction at 180 min ($P=0.017$), but not at 60 min ($P=0.338$; Fig. 1A). In obese hyperinsulinemic volunteers (Table 1), whose AUC for glucose and insulin was significantly higher than those of normoweight volunteers (Table 1), baseline fasting anandamide, but not 2-AG, levels were significantly higher than in normoweight/normoinsulinemic volunteers, and did not change significantly following oral glucose either at 60 or 180 min (Fig. 1B). Also, the decrease in 2-AG levels at 180 min in this case did not reach statistical significance (Fig. 1B). When pooling together both normoweight and obese volunteers, the baseline fasting levels of anandamide correlated positively with BMI ($r^2=0.68$; $P<0.0001$), waist circumference ($r^2=0.66$; $P<0.0001$), VAT ($r^2=0.65$; $P<0.0001$), SAT ($r^2=0.50$; $P=0.0005$), and TG levels ($r^2=0.44$; $P=0.0018$). By contrast, 2-AG levels correlated positively only with TG levels ($r^2=0.46$;

Figure 1 Effect of OGTT on anandamide and 2-arachidonoyl-glycerol (2-AG) plasma levels in normoweight/normoinsulinemic volunteers (A) and obese/hyperinsulinemic volunteers (B) (Table 1 for volunteers data). Data are expressed as means $\pm$ S.E.M. of $n=10$ in each group and were compared by means of repeated-measures ANOVA. In (B) data for anandamide were compared with the corresponding values in (A) by the Student–Newman–Keuls's test (P values shown on each bar).

$P=0.03$), and only in obese individuals. The normalized (Δ /baseline) oral glucose-induced changes of anandamide levels at 60 and 180 min correlated negatively with BMI ($r^2=-0.23$; $P=0.031$ and $r^2=-0.38$; $P=0.0037$ respectively), waist circumference ($r^2=-0.28$; $P=0.016$ and $r^2=-0.44$; $P=0.0014$ respectively), and SAT ($r^2=-0.28$; $P=0.015$ and $r^2=-0.33$; $P=0.0077$ respectively), and, at 180 min only, with fasting insulin levels ($r^2=-0.38$; $P<0.0035$) and fasting glucose levels ($r^2=-0.21$; $P<0.041$). Importantly, the Δ /baseline values for anandamide at both 60 and 180 min correlated negatively with both AUC's for glucose ($r^2=-0.31$; $P=0.01$ and $r^2=-0.21$; $P=0.04$ respectively) and, particularly, AUC's for insulin ($r^2=-0.36$; $P=0.005$ and $r^2=-0.46$; $P=0.001$ respectively). No such correlations were found for the normalized OGTT-induced changes of 2-AG levels, except for a trend with fasting insulin at 180 min ($r^2=-0.18$; $P=0.061$).

Effect of acute insulin infusion on EC levels in male volunteers

The cohort employed for this second part of the study (Table 2) was composed of 19 male volunteers with a normoweight/overweight borderline BMI, with or without T2D and, in some cases, with elevated fasting insulin levels and liver fat and reduced HDL cholesterol levels. However, no statistically significant difference was observed between the anandamide or 2-AG basal plasma levels of nondiabetic and T2D volunteers at baseline (Fig. 2A), nor at 240 and 480 min postsaline infusion (data not shown). The basal levels of 2-AG correlated positively with TG levels ($r^2=0.21$; $P<0.05$) and ApoB levels ($r^2=0.21$; $P=0.05$). No correlation was found between the basal levels of anandamide and any of the anthropometric or biochemical parameters measured.

The insulin infusion caused a decrease in anandamide, but not 2-AG, plasma levels (Fig. 2B). With repeated-measures ANCOVA, using baseline values as a covariate, we found for anandamide a P value <0.0001 for $\text{TIME} \times \text{INSULIN}$, whereas, for 2-AG, the P value was 0.367. A significant difference was also observed between the insulin infusion-induced Δ 's of EC levels of nondiabetic and T2D volunteers at 240 min post-infusion, with a significantly smaller decrease being observed for anandamide (mean Δ from 1.94 ± 0.30 to 1.07 ± 0.43 pmol/ml, $P=0.027$), and a negative decrease for 2-AG (mean Δ from 1.03 ± 1.50 to -4.60 ± 3.02 pmol/ml, $P=0.019$), in T2D patients (Fig. 2C).

In this second study, only weak correlations were found between insulin infusion-induced Δ 's of blood EC levels and parameters of insulin resistance, hepatic fat and inflammation, dyslipidemia, and dyslipoproteinemia. Thus, for example, insulin infusion-induced decreases in anandamide levels correlated negatively with fasting insulin at 240 min ($r^2=-0.21$; $P=0.044$); with TG levels at both 240 and 480 min ($r^2=-0.21$; $P=0.047$ and $r^2=-0.23$; $P=0.039$ respectively); with ALAT at 240 min ($r^2=-0.27$; $P=0.024$); and with ApoB levels at 480 min ($r^2=-0.26$; $P=0.024$); but positively with HDL cholesterol levels at both 240 and 480 min ($r^2=0.27$; $P=0.021$ and $r^2=0.27$; $P=0.024$ respectively). The insulin infusion-induced Δ 's of 2-AG levels correlated only with fasting insulin at 240 min ($r^2=-0.25$; $P=0.030$). No stronger correlations were found when using instead normalized insulin infusion-induced changes (Δ/saline) of EC levels. In the case of anandamide, these correlated negatively with TG levels at both 240 and 480 min ($r^2=-0.24$; $P=0.031$ and $r^2=-0.28$; $P=0.02$ respectively); with fasting insulin, liver fat, and ALAT at 240 min ($r^2=-0.22$; $P=0.043$, $r^2=-0.23$; $P=0.038$; and $r^2=-0.25$; $P=0.028$ respectively); and with intra-abdominal fat at 480 min ($r^2=-0.24$; $P=0.04$); but positively with HDL

Figure 2 Basal fasting anandamide and 2-arachidonoylglycerol (2-AG) plasma levels in normoweight/overweight borderline male volunteers with ($n=7$) and without ($n=12$) type 2 diabetes (A), their changes (B) in the whole cohort ($n=19$) at 240 and 480 min following saline infusion (Sal) or insulin infusion clamp (Ins), and (C) their decreases at 240 min following saline insulin versus saline infusion in either nondiabetic or type 2 diabetics, taken separately. In (A), blood samples were taken 30 min prior to insulin or saline infusion. Data are expressed as means $\pm$ S.E.M. and were compared between diabetic and nondiabetic by the Student–Newman–Keuls’s test in (A). In (B), saline and clamp curves were compared, with correction of the baseline values (before the clamp) using repeated-measures ANCOVA and, for AEA, $^aP<0.0001$ for $\text{TIME} \times \text{INSULIN}$ was found, whereas, for 2-AG, the difference was not significant ($P=0.367$). In (C) changes were compared by the Student–Newman–Keuls’s test.

cholesterol levels at both 240 and 480 min ($r^2=0.28$; $P=0.02$ and $r^2=0.30$; $P=0.016$ respectively). On the other hand, the normalized insulin infusion-induced changes of 2-AG levels did not correlate with any of the anthropometric or biochemical parameters measured.

Discussion

Previous data suggest that both glucose and insulin may affect EC biosynthesis and degradation in isolated cells (12, 16, 17). Therefore, in this study, we aimed at gaining direct evidence, in intervention studies carried out in two different cohorts of volunteers, of potential effects of either oral glucose load or insulin infusion on plasma EC levels. We first wanted to understand whether, during oral glucose load, the subsequent elevation of glucose and insulin plasma levels is accompanied by changes in plasma EC levels in normoweight and normoinsulinemic men and women. Despite the fact that, in isolated cells, glucose can cause elevation of EC levels (12, 17), we found here a strong decrease in EC levels at both 60 and, particularly, 180 min of the OGTT. The decreased blood EC levels observed following OGTT might suggest that insulin exerts a negative effect on plasma EC levels, as previously suggested in studies with isolated adipocytes and β -cells (12, 17). In agreement with this hypothesis, we observed that the levels of either anandamide or 2-AG did not decrease significantly during the OGTT in obese hyperinsulinemic volunteers who were insulin resistant. This finding is in agreement with the lack of stimulatory effect of insulin on FAAH expression in adipocytes from obese individuals (16), since lack of FAAH upregulation should cause lack of anandamide downregulation.

To further investigate the nature of the oral glucose load-induced decreases in EC levels, we evaluated the correlations of these changes with various anthropometric and metabolic parameters that had been measured in the two study groups. In agreement with the possible role of insulin sensitivity as a determinant for decreases in EC levels, we found that the decreases in anandamide observed at 180 min were negatively correlated with fasting insulin and glucose levels. More importantly, the OGTT-induced decreases in anandamide levels always strongly and negatively correlated with the AUC values for insulin, which are a very strong predictor of insulin resistance. The decreases in anandamide levels at both 60 and 180 min during the OGTT correlated negatively also with waist circumference, BMI, and SAT.

VAT, but not SAT, was previously shown to strongly correlate with plasma 2-AG, but not anandamide, levels in cross-sectional and intervention studies carried out in obese men with a much higher (at least twofold) average VAT/SAT ratio than that observed even in the obese volunteers employed in the present study

(10, 11). The significantly lower degree of VAT in the present cohort, which also included women, might also explain why, in contrast to the previous observations in abdominally obese men (9–11), the present subjects exhibited higher baseline levels of anandamide, but not of 2-AG, than normoweight volunteers, and why only baseline anandamide levels in both groups correlated positively with BMI, waist circumference, VAT, and SAT. In our volunteers, however, we did confirm the strong direct correlation between 2-AG, but not anandamide, baseline levels, and TG levels (9–11). It must be pointed out that anandamide and 2-AG tissue and plasma levels have been found very often to change in different ways in several physiological and pathological conditions, and in both laboratory animals and humans. This probably reflects the fact that the two compounds might play different roles, as anandamide, for example, is known to interact also with noncannabinoid receptors (28).

Since the results in this cohort study suggested a possible role of insulin in controlling peripheral EC levels, we aimed at gaining direct evidence of this by studying the effect of insulin infusion on EC levels utilizing the euglycemic clamp. Given the significant correlations between BMI and OGTT-induced decreases in anandamide levels observed in the first cohort, we chose to perform the insulin clamps in a cohort of volunteers with a wide range of BMI (from 22.2 to 35.3 kg/m², with mean BMI = 25.8 kg/m²). This strategy aimed at reducing the potential impact of BMI, which is not a strong correlate of insulin resistance, but is still associated with high plasma EC levels (9, 19). In this cohort of 19 individuals, strong differences existed in terms of dyslipidemia, liver fat, HDL cholesterol and of the presence of T2D, which we thought would allow us to speculate about the possible role of these factors as predictors of plasma EC levels and their changes following EHC.

In agreement with the results of the first study, we found that plasma anandamide levels were decreased during insulin infusion as compared with saline infusion, at both 240 and 480 min postinfusion. We also found that significantly smaller insulin-induced decreases in anandamide levels at 240 min postinfusion were observed in T2D patients, whereas 2-AG levels even tended to increase. This finding was not surprising in view of the fact that T2D patients have little residual insulin sensitivity. These data strongly suggest that insulin can be a negative regulator of blood 2-AG and, particularly, anandamide levels in only insulin-sensitive subjects.

Interestingly, the presence of T2D *per se* (and the subsequent hyperglycemia), unlike what has previously been observed in overweight/obese (BMI ~ 33) T2D patients of older age (65.7) and mixed gender, but with comparable elevation of triglyceridemia (2.0 nmol/l), fasting glucose, and total cholesterol (12), was not associated with elevated fasting EC levels, suggesting

that the copresence of BMI > 28 and/or older age is necessary to observe a stronger basal EC tone in T2D (12). However, since T2D patients are clearly also insulin resistant, it is reasonable to suggest, based on the present data, that they are likely to always exhibit higher postprandial anandamide levels.

When we attempted to correlate, in the whole cohort, some important predictors of insulin resistance and/or dyslipidemia, i.e. high fasting insulin, TGs, liver fat and ApoB, and low HDL cholesterol, with the capacity of insulin to lower EC plasma levels at 240 and/or 480 min postinfusion, we found negative as well as weak correlations for anandamide, whereas insulin infusion-induced changes in 2-AG levels correlated negatively only with fasting insulin, and only at 240 min postinfusion. This suggests that, even in nonobese men, a postprandial overactivity of the peripheral EC system might be determined, perhaps not by insulin resistance, fatty liver, and dyslipidemia individually, but at least by their concomitant presence.

We also found that, even in this cohort of men with moderate intra-abdominal adiposity, fasting 2-AG levels correlate with TG (and ApoB) levels, as shown above and previously in obese patients (9–11), whereas no such correlation is seen with fasting anandamide levels. Thus, in these men, while TG and ApoB appear to be associated with high 2-AG levels independently of the effects of insulin, these same metabolic factors as well as high liver fat and fasting plasma insulin, and low HDL cholesterol, are weakly associated only with impaired insulin-induced decreases of anandamide levels.

In conclusion, we provided here novel evidence suggesting that insulin negatively controls plasma EC, and particularly anandamide, levels, which, in turn, might reflect EC spillover from peripheral organs. This control seems to be impaired, with subsequent postprandial EC overactivity, not only in obese individuals with reduced insulin sensitivity, but also in nonobese men with fatty liver and dyslipidemia, to an extent mostly independent from their BMI. If future studies will demonstrate that the dysregulated peripheral EC system is one of the underlying causes of insulin resistance, hepatic steatosis, and dyslipidemia, rather than their consequence, the present findings might improve our understanding of the aetiopathology of these metabolic disorders and, hence, open new avenues in their pharmacological treatment (29–31).

Declaration of interest

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