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EFEITO DA EXPRESSÃO DA PROTEÍNA DE TRANSFERÊNCIA DE COLESTERIL ÉSTER (CETP) SOBRE O METABOLISMO DAS LIPOPROTEÍNAS RICAS EM TRIGLICÉRIDES E ADIPOSIDADE EM CAMUNDONGOS TRANSGÊNICOS.

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Funcional e Molecular, na área de Fisiologia.

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RESUMO

Neste trabalho investigamos o efeito da expressão de genes envolvidos no transporte e redistribuição de triglicérides das lipoproteínas plasmáticas, a apolipoproteína (apo) CIII e a proteína de transferência de colesteril éster (CETP), sobre o metabolismo de triglicérides pós-prandial e sobre a formação de depósitos adiposos regionais em camundongos geneticamente modificados. Podemos resumir nossos achados da seguinte maneira: a expressão da CETP leva ao aumento da trigliceridemia pós prandial; nenhuma alteração da absorção intestinal de gorduras e de secreção hepática de VLDL (lipoproteína de densidade muito baixa); redução da atividade da lipoproteína lipase e retardo na remoção plasmática de lipoproteínas ricas em triglicérides (TG). Mediante longo prazo de dieta rica em gordura, a super-expressão da CETP também provoca redução da gordura subcutânea, redução do tamanho do adipócito e da concentração plasmática de leptina em camundongos transgênicos hipertrigliceridêmicos que super-expressam a apo CIII. Por outro lado, a super-expressão de apo CIII não afeta o tamanho dos depósitos adiposos viscerais e subcutâneos na vigência de dieta pobre em gordura, porém causa aumento dos depósitos adiposos viscerais e subcutâneos e do tamanho dos adipócitos viscerais e concentração de leptina mediante dieta rica em gordura. Os camundongos que super-expressam a apo CIII não apresentaram diferenças na adiposidade quando sob dieta pobre em gordura devido a um aumento do metabolismo corporal associado a maior velocidade de respiração mitocondrial de repouso. Porém, quando submetidos à dieta rica em gordura, acumulam mais tecido adiposo visceral e subcutâneo, tornando-se mais obesos que os controles. A expressão da CETP neste contexto metabólico de hipertrigliceridemia neutraliza o efeito adipogênico da apo CIII.

ABSTRACT

Cholesteryl ester transfer protein (CETP) promotes the exchange between cholesteryl ester (CE) from HDL and triglycerides (TG) from TG rich lipoproteins. The overexpression of apolipoprotein (apo) CIII in transgenic mice causes hypertriglyceridemia due to decreased TG rich lipoprotein plasma removal rate. In this work we investigated whether CETP expression and apo CIII expression affect the post-prandial TG levels and diet induced visceral adipose tissue formation in genetically modified mice. Results showed that the expression of CETP lead to augmented post-prandial TG levels, similar intestinal fat absorption and hepatic TG and cholesterol secretion rates, diminished TG rich lipoproteins plasma removal rates and reduced lipoprotein lipase activity. These findings indicate that the levels of circulating CETP modulate dietary fat tolerance. Under long-term high fat diet, the expression of CETP reduced the subcutaneous adipose depot, visceral adipocyte size and plasma leptin levels of hypertriglyceridemic mice overexpressing the apo CIII. On the other hand, under low fat diet, the apo CIII transgenic mice presented visceral and subcutaneous adipose depots similar to the wild type mice and increased body metabolic rate and mitochondrial resting respiration rates. However, under high fat diet, the apo CIII transgenic mice showed increased visceral and subcutaneous adipose tissue, visceral adipocyte size and plasma leptin levels and no differences in body energy dissipation (rectal temperature and mitochondrial resting respiration). In conclusion, the elevation of plasma apo CIII levels aggravates diet-induced obesity and the expression of physiological levels of circulating CETP reverses this adipogenic effect, indicating a novel role for CETP in modulating adiposity.

LISTA DE ABREVIATURAS

ABCA1, ABCG1, ABCG1 - *ATP-binding cassete transporter-A1, G1, G4, respectivamente*

AGL - Ácidos graxos livres

apoAI, apoCIII, apoE - Apolipoproteína AI, CIII, E, respectivamente

CE - Colesteril éster, colesterol esterificado

CL - Colesterol livre

CETP - Proteína de transferência de colesteril éster

COL - Colesterol

DAC - Doença arterial coronariana

HDL - Lipoproteína de alta densidade

HMG-CoA - 3-hidroxi-3-metil-glutaril coenzima A redutase

IDL - Lipoproteína de densidade intermediária

KDa - Quilodaltons

Kb - Quilobase

LCAT - Lecitina: colesterol acil transferase

LDL - Lipoproteína de baixa densidade

LH - Lipase hepática

LP - Lipoproteína

LPL - Lipoproteína lipase

LRP- *LDL receptor related protein*

PL - Fosfolipídes

QM - Quilomícrons

SRA – Receptores *scavenger*, tipo A; SR-B1 - Receptores *scavenger*, classe B tipo 1

Tg - Transgênicos

TG - Triglicérides

TRC - Transporte reverso do colesterol

UCP - *Uncoupling protein*

VLDL - Lipoproteína de densidade muito baixa

SUMÁRIO

I - INTRODUÇÃO

Nas últimas décadas a ingestão de gordura tem recebido considerável atenção visto que seu aumento está associado ao aumento dos riscos de doenças coronárias (Taubes, 2001) e de obesidade e suas co-morbidades (Hill *et al.*, 2000). As dietas ricas em ácidos graxos saturados são mais potentes em aumentar o colesterol sanguíneo do que as dietas ricas em ácidos graxos poli-insaturados (Beynem & Kritchevsky, 1986). A hipercolesterolemia, seja de origem dietética ou genética, está diretamente correlacionada ao desenvolvimento de aterosclerose (Kannel, 1985; Goldstein & Brown, 1987). A recomendação da *American Heart Association* é que o consumo total de gordura deve ser 30% ou menos das calorias totais ingeridas, sendo menos de 10% na forma de gordura saturada. A hipertrigliceridemia está frequentemente associada com outros fatores de risco de doença vascular, como a resistência à insulina, hipertensão, obesidade visceral e redução nos níveis de HDL-colesterol (Grundy, 1998). Além disso, há evidências demonstrando que as concentrações plasmáticas de triglicérides também são fatores de risco diretos ou indiretos para o desenvolvimento de doença aterosclerótica (Fontbonne *et al.*, 1989; Castelli, 1992; Gotto, 1998; Malloy & Kane, 2001; Kawakami & Yoshida, 2005).

Neste trabalho estudamos os efeitos da super-expressão de proteínas envolvidas no transporte de colesterol e de triglicérides (CETP e apolipoproteína CIII) sobre o metabolismo de triglicérides a curto-prazo (pós-prandial) e a longo prazo, sobre a formação de tecido adiposo induzida por dieta rica em gordura.

1- Metabolismo de lipoproteínas

A homeostase do colesterol e dos triglicérides no compartimento plasmático e nos tecidos é regulada por processos fisiológicos complexos que envolvem a síntese e secreção de lipoproteínas, atividade de receptores celulares específicos para as lipoproteínas, atividade de enzimas lipolíticas e de proteínas de transferência de lípides.

As lipoproteínas são partículas de tamanho e densidade variáveis, responsáveis por transportar substâncias lipossolúveis provenientes da dieta ou sintetizadas pelo próprio organismo. No núcleo das partículas concentram-se os componentes triglicérides (TG), colesterol esterificado, e vitaminas lipossolúveis, envolvidos por uma camada de fosfolipídes, colesterol livre e proteínas específicas, as apolipoproteínas (apo), as quais

mantém sua estabilidade e solubilidade (Brown *et al.*, 1981).

Há cinco classes principais de lipoproteínas plasmáticas (LP) transportadoras de lipídeos, classificadas de acordo com a sua densidade: as LP de alta, baixa, intermediária e muito baixa densidade, respectivamente, HDL, LDL, IDL e VLDL, além dos quilomícrons (QM). As várias LP diferem entre si em termos de densidade, tamanho e migração eletroforética porque têm composição lipídica e protéica distintas. QM e VLDL são as chamadas LP ricas em triglicérides, de origem intestinal e hepática, respectivamente.

Quando na circulação sistêmica, os TG contidos nos quilomícrons sofrem hidrólise pela ação da lipoproteína lipase (LPL), enzima que se encontra ancorada na membrana basal das células endoteliais dos vasos sanguíneos dos vários órgãos (Bisgaier & Glickman, 1983). Nesse processo, além de perder TG, os quilomícrons perdem também colesterol livre (CL), fosfolipídes (PL) e apoA para as HDL circulantes e ganham apoE e apoC, resultando em uma partícula denominada remanescente de quilomícrons, pobre em TG e rica em colesterol esterificado (CE) e apoE.

As apoE dos remanescentes de quilomícrons são reconhecidas por receptores hepáticos com alta afinidade, os quais promovem uma rápida captação destes pelos hepátocitos (Tall *et al.,* 1979; Havel, 1986; Mahley, 1998). Esses receptores são denominados receptores de remanescentes de quilomícrons ou receptor E ou ainda LRP (*LDL receptor-related protein*) (Herz *et al.*, 1988). Esse sistema é de alta eficiência resultando em uma meia-vida da partícula na circulação muito curta, nos seres humanos de aproximadamente 10 minutos (Redgrave*,* 1983; Cooper *et al.*, 1997).

As VLDL são secretadas pelo fígado e passam pelas mesmas etapas iniciais do catabolismo vascular dos quilomícrons, porém com menor velocidade. Diminuem de tamanho e geram os remanescentes de VLDL ou IDL, os quais praticamente só contém apoB100 e apoE como componentes protéicos. Grande parte das IDL (~75%) é rapidamente captada via receptores hepáticos específicos (LRP e receptores de LDL) e o restante continua circulando, perdendo mais TG e PL pela ação da lipoproteína lipase periférica e hepática (LH), transformando-se então em LDL (Brown & Goldstein, 1981, Fielding & Fielding, 1991).

As LDL contêm basicamente apoB100 e colesterol esterificado. Seu *turnover* é mais lento (cerca de 2 dias) e assim como as IDL, também são removidas da circulação por receptores hepáticos que reconhecem sítios idênticos na cadeia da apoE e da apoB100.

Esses receptores foram chamados de receptores B-E ou receptores de LDL (Brown & Goldstein, 1986).

Concentração elevada de LDL plasmática resulta em aumento da quantidade de LDL que se infiltra no espaço subendotelial. A LDL interage com elementos da matriz extracelular (ex.: glicosaminoglicanos e colágenos) e fica retida por mais tempo neste microambiente, onde sofre alterações químicas tais como oxidação e proteólise. Esta LDL quimicamente modificada estimula as células endoteliais a expressarem moléculas de adesão facilitando a adesão e transmigração de células linfomonocitárias circulantes para o espaço subendotelial. Os monócitos proliferam-se e diferenciam-se em macrófagos que captam as LDL modificadas via receptores *scavenger* do tipo A (SRA). Diferentemente dos receptores de LDL, os receptores *scavenger* não são regulados e os macrófagos captam as LDL modificadas de maneira contínua transformando-se em *foam cells*. Com o tempo, as *foam cells* morrem e vão constituir o núcleo necrótico da lesão aterosclerótica avançada. Células musculares lisas também migram da camada adventícia para íntima, proliferam-se e eventualmente transformam-se em *foam cells*. O processo ocorre em progressão geométrica com a participação de um grande número de fatores de crescimento, citocinas e moléculas vasoregulatórias (Ross, 1993; Lusis, 2000; Libby, 2000).

Por outro lado, a concentração plasmática elevada de HDL está associada à redução de risco de aterosclerose. Uma das teorias que explica o papel atero-protetor da HDL é o chamado transporte reverso de colesterol (TRC), eficiente mecanismo para descartar o excesso de colesterol dos tecidos através da excreção pelo fígado. Este sistema envolve o efluxo de colesterol livre das membranas celulares, via transportador ABCA1 (*ATP binding cassete transporter*) (Dean *et al.*, 2001; Oram *et al.*, 2000), para as apo-A1 pobre em lipídios. Na mesma família de transportadores, os ABCG1 e ABCG4 promovem o efluxo de colesterol das células para as HDL (Wang *et al.*, 2004). Os passos subsequentes do TRC são a esterificação do colesterol pela LCAT (*lecithin-cholesterol acyl transferase*) (Glomset, 1968) e a transferência do colesteril éster (CE) para lipoproteínas ricas em TG mediada pela CETP (Hesler *et al.*, 1987; Tall *et al.*, 2000). Posteriormente, o CE pode ser transferido para o fígado diretamente da HDL através dos receptores SR-B1 (Acton *et al.*, 1996; Landschulz *et al.*, 1996) e indiretamente das LP que contem apo-B, através do receptor de LDL (Brown & Goldstein, 1986) e do LRP (Herz *et al.*, 1988). A ação da CETP na HDL facilita a velocidade de efluxo de colesterol da célula. Também a ação combinada

da CETP e lipase hepática na HDL_2 madura, regenera HDL_3 e pré-beta-HDL, subfrações que são melhores aceptoras de colesterol das células (Bruce *et al.*, 1998).

As HDL têm ainda outras propriedades anti-aterogênicas, tais como ação anti-inflamatória, anti-oxidante e anti-trombótica (Stein & Stein, 1999).

2- Proteína de transferência de colesteril éster (CETP)

A CETP é uma glicoproteína constituída de 476 aminoácidos, dos quais cerca de 40% são resíduos hidrofóbicos. Seu peso molecular aparente é de 66 a 74 KDa. Seu gene situa-se no braço longo do cromossomo 16 e consiste de 16 exons e 15 introns, perfazendo um total de 25 Kb de DNA genômico (Tall, 1995).

A atividade da CETP já foi documentada em mais de 16 espécies de vertebrados, incluindo peixes, répteis, aves e mamíferos. Ratos e camundongos não expressam a CETP, enquanto uma alta atividade é observada no plasma de coelhos (Ha & Barter, 1982). Em humanos, a CETP é sintetizada predominantemente no fígado, baço, e tecido adiposo, com menores níveis de expressão no intestino delgado, adrenal, rim, músculo esquelético e coração (Tall, 1995). Nos macacos, os níveis plasmáticos de CETP correlacionam positivamente com a expressão hepática de RNAm da CETP, sendo o fígado a principal fonte de secreção desta proteína (Quinet *et al.*, 1991). No hamster, a maior expressão da CETP é detectada no tecido adiposo, músculo, e intestino delgado, sendo pouco expressa no fígado (Jiang *et al.*, 1991). O tecido adiposo parece ser um local que conserva alta expressão da CETP em muitas espécies. A concentração plasmática de CETP é positivamente correlacionada com os níveis de RNAm da CETP do tecido adiposo nos hamsters (Quinet *et al.*, 1993) e nos macacos (Quinet *et al.*, 1991). Camundongos com expressão de CETP em vários tecidos (promotor natural da CETP) e camundongos com expressão exclusiva no tecido adiposo (promotor da aP2) apresentam a mesma quantidade de CETP circulante (Zhou *et al.*, 2006).

Nos humanos, a CETP é expressa em grandes quantidades no tecido adiposo (Radeau *et al.*, 1998) e, em cultura de células, a CETP é expressa nos estágios iniciais da adipogênese, antes mesmo do aparecimento dos fatores de transcrição das famílias PPAR, SREBP e C/EBP (Gauthier *et al*., 1999). Outros estudos com seres humanos mostram que a atividade da CETP correlaciona com o grau de obesidade (Dullaart *et al.*, 1994, Arai *et al.*, 1994), e que a redução de peso está associada à diminuição da atividade de CETP

plasmática (Dullaart *et al.*, 1994).

Em 2000, Dusserre *et al.* demonstraram que, em humanos, a expressão da CETP é maior no tecido subcutâneo que no visceral. No tecido adiposo subcutâneo, a expressão do mRNA da CETP é mais alta em adipócitos pequenos e com menor quantidade de gotículas lipídicas (Radeau *et al.*, 1995) e diminui com a idade (Radeau *et al*., 1998).

A expressão gênica da CETP pode ainda ser regulada por estímulos nutricionais, hormonais e inflamatórios (Tall, 1993; 1995).

Em uma variedade de espécies como coelhos (Quinet *et al.*, 1990), macacos (Quinet *et al.*, 1991), hamsters (Jiang *et al.*, 1991), camundongos transgênicos (Jiang *et al*., 1992) e seres humanos (Martin *et al*., 1993), observou-se aumento do mRNA da CETP em resposta à dieta rica em colesterol. A hiperlipemia endógena pode induzir aumento da expressão do gene da CETP por um mecanismo similar ao observado com dieta rica em colesterol (Tall, 1995). Oliveira *et al* (1996) localizaram a região responsiva ao colesterol no promotor do gene da CETP, enquanto Luo & Tall (2000) mostraram que o transativador LXR (*Liver X receptor*) era o responsável pela ativação do gene da CETP na vigência da dieta rica em colesterol.

A hiperinsulinemia produzida durante o procedimento de *clamp* euglicêmico-hiperinsulinêmico diminui a atividade da CETP em diabéticos do tipo 2 (Sutherland *et al.*, 1994). Berti *et al.* (2003) mostraram que a insulina regula negativamente a expressão da CETP. Hormônios da tireóide também podem alterar a expressão da CETP. Tan *et al.* (1998) verificaram uma diminuição da atividade da CETP em pacientes hipotireoideos e um aumento dos pacientes hipertireoideos, quadro que foi revertido com o tratamento para restabelecer a função tireoideana. Em estudos com camundongos transgênicos para a CETP, observou-se que o excesso de hormônio tireoideano aumentou a atividade plasmática da CETP, enquanto no hipertireoidismo não houve alteração da CETP (Berti *et al.*, 2001).

A expressão do gene da CETP é influenciada também pelos corticoesteróides. Tratamento com corticoesteróides em seres humanos e camundongos transgênicos para a CETP induziu diminuição das concentrações plasmáticas da CETP (Tall, 1995). Nos camundongos transgênicos para CETP esse efeito foi mediado por uma diminuição do mRNA da CETP no fígado (Masucci-Magoulas *et al.*, 1996).

A CETP medeia toda a atividade de troca de lipídeos neutros no plasma,

transferindo os triglicérides das VLDL e quilomícrons para as HDL, e o colesteril éster no sentido oposto, das HDL para as lipoproteínas ricas em TG. Este processo de transferência é bidirecional e não equimolar (Oliveira & Quintão, 1996). Portanto, a CETP promove uma redistribuição dos TG entre LP que são hidrolisadas em compartimentos vasculares distintos, i.e., HDL pela lipase hepática nos sinusóides hepáticos e LP ricas em TG hidrolisadas pela lipase lipoprotéica periférica nos tecidos extra-hepáticos, principalmente músculo e tecido adiposo.

A CETP tem uma função importante no transporte reverso de colesterol aumentando o movimento do colesterol do plasma para o fígado via transporte reverso de colesterol indireto. Essa proteína também pode estimular a transferência do colesterol de tecidos periféricos para as HDL (primeira etapa do transporte reverso de colesterol) de duas maneiras: 1- atuando em conjunto com a lipase hepática na reconstituição de partículas pequenas de HDL (HDL_3 e préβ HDL), as quais são mediadores ótimos do efluxo celular de colesterol (Bruce *et al.*, 1998; Kunitake *et al.*, 1992) e 2 - estimulando a ação da LCAT (Oliveira *et al.,* 1996). Gauthier *et al.*, (2005) demonstraram que a CETP tem um papel direto na captação seletiva do CE das HDL pelos hepatócitos, independentemente de outros receptores de lipoproteínas.

A deficiência genética da CETP foi documentada pela primeira vez em 1985 por Kurasawa & Koizumi e seus colaboradores. Desde então, mutações e polimorfismos nas regiões codificadoras e regulatórias do gene da CETP tem sido descritos e sua relação com aterosclerose investigada em populações asiáticas, caucasianas, afro-americanas e outras (Nagano *et al.*, 2004; Thompson *et al.*, 2004). O papel pró ou anti-aterogênico da CETP é objeto de intenso debate na literatura (Hirano *et al*., 2000).

Em seres humanos, a deficiência genética de CETP acompanhada de altas concentrações de HDL-colesterol (> 60 mg/dL) está estatisticamente correlacionada a um menor risco de doença arterial coronariana (DAC). No entanto, indivíduos com deficiência de CETP com níveis moderadamente altos de HDL (40-60 mg/dL) apresentam maior risco de DAC (Zhong *et al.*, 1996). Estudos com inibidores da CETP têm sido considerados como terapia anti-aterogênica (Okamoto *et al.*, 2000; Huang *et al.*, 2002; Barter *et al.*, 2003, Clark *et al.*, 2004; Brousseau *et al.*, 2004; Davidson *et al.,* 2006).

Durante os últimos anos, estudos com camundongos geneticamente modificados mostraram que a ação da CETP sobre a aterogênese é claramente dependente do contexto

metabólico. O papel protetor da CETP foi demonstrado em camundongos transgênicos expressando a CETP em condições de hipertrigliceridemia (Hayek *et al.*, 1995), na superexpressão da LCAT (Foger *et al.*, 1999; Berti *et al.*, 2005 a), na superexpressão da apoB combinada com deficiência da lipoproteína lipase e diabetes (Kako *et al.*, 2002), em camundongos castrados (Cazita *et al.*, 2003; Casquero *et al.*, 2006) e em camundongos machos diabéticos db/db (MacLean *et al.*, 2003). Contudo, outros autores observaram ações pró-aterogênica em camundongos que expressam a CETP em concentrações supra-fisiológicas (Marotti *et al.*, 1993), em hipercolesterolemia severa por ausência completa de receptores LDL ou da apo E (Plump *et al.*, 1999) e em ratos hipertensos (Herrera *et al.*, 1999). Há ainda situações onde a expressão da CETP é neutra para o desenvolvimento de aterosclerose (Foger *et al.*, 1999; Berti *et al.*, 2005 b).

3- Lipoproteínas ricas em triglicérides, apolipoproteína CIII e hipertrigliceridemia

Zilversmit (1979) propôs que a aterogênese é um fenômeno pós-prandial. Desde então, a hiperlipidemia pós-prandial, e em especial o metabolismo dos remanescentes das lipoproteínas ricas em TG vem sendo um importante foco de investigação científica. O debate perdurou por mais de uma década, quando a hipertrigliceridemia era apontada apenas como um marcador de risco, uma vez que esta frequentemente era associada a outros fatores de risco como a resistência à insulina, hipertensão, obesidade visceral e redução nos níveis de HDL-colesterol (Grundy, 1998). Mais recentemente verificou-se que o elevado nível de triglicérides no sangue é fator de risco independente para o desenvolvimento de doença aterosclerótica (Kawakami & Yoshida, 2005, Malloy & Kane, 2001).

Nos países industrializados, os indivíduos fazem no mínimo três refeições diárias e desta maneira permanecem em estado pós-prandial praticamente por todo dia (Wilhelm & Cooper, 2003). Os lípides absorvidos da dieta são incorporados aos quilomícrons e os ácidos graxos derivados de seus TG são armazenados no tecido adiposo ou utilizados de maneira imediata pelos tecidos. A lipemia pós-prandial é um fenômeno fisiológico, mas quando há excesso de gorduras provenientes da dieta pode ocorrer tanto acúmulo de partículas remanescentes aterogênicas no plasma (Karpe, 2002), quanto à indução de maior formação de reserva adiposa. A magnitude da lipemia pós-prandial depende do conteúdo de gordura nas refeições e pode ser aumentada por consecutivas refeições ricas em gordura

(Jackson *et al.*, 2002).

A presença de DAC está associada aos maiores níveis de triglicérides plasmáticos no estado pós-prandial dos indivíduos afetados comparados aos controles saudáveis (Groot *et al.*, 1991, Van Oostrom *et al.*, 2004). Esta anormalidade pode explicar por que alguns indivíduos desenvolvem DAC mesmo com níveis normais de lipídeos no estado de jejum (Kolovou *et al.*, 2005). Alterações no metabolismo das lipoproteínas na fase pós-prandial são bastante observadas nos indivíduos diabéticos tipo 2 (Curtin *et al.*, 1994), em heterozigotos para hipercolesterolemia familiar (Kolovou *et al.*, 2005), em hipertensos (Kolovou *et al.*, 2003) e outras condições (Kolovou *et al.*, 2003).

As LP ricas em TG, bem como seus remanescentes, podem estar envolvidas diretamente na aterogênese através da indução de estresse oxidativo, de toxicidade endotelial, de condições protrombóticas, de estimulação da expressão endotelial de moléculas de adesão celular e alteração do tônus vascular por redução da produção de óxido nítrico (para revisão ver Wilhelm & Cooper, 2003). Steiner (1998) sugere ainda que as LP ricas em TG estejam envolvidas nos estágios iniciais da aterogênese.

Estudos prévios têm demonstrado que a apolipoproteína CIII (apo CIII) pode contribuir para o desenvolvimento da hipertrigliceridemia na população humana. Correlação positiva tem sido observada entre os níveis plasmáticos de apo CIII e elevados níveis plasmáticos de TG (Jong *et al.*, 2001) e de VLDL-TG (Le *et al.*, 1988). A apo CIII é uma proteína de 8 a 9 KDa, sintetizada no fígado e em menor quantidade no intestino, secretada como constituinte das lipoproteínas ricas em TG e da HDL. A manipulação genética em camundongos revelou que as apo CIII provocam alterações do metabolismo de TG, pois sua super-expressão leva a hipertrigliceridemia (Ito *et al.*, 1990; Aalto-Setala *et al.*, 1992) e a sua deficiência leva à hipotrigliceridemia (Maeda *et al.*, 1994). Várias evidências clínicas e experimentais sugerem que a apo CIII inibe a ação da lipoproteína lipase e a captação tecidual das LP ricas em TG (Wang *et al.*, 1985; McConathy *et al.*, 1992; Mann *et al.*, 1997; Ebara *et al.*, 1997; Jong *et al.*, 1999). A apo CIII é a mais abundante da família das apolipoproteínas Cs, que inclui as apo CI e CII. Estas últimas, quando super-expressas, também levam à hipertrigliceridemia (Schachter *et al.,* 1994 e 1996).

Os camundongos que super-expressam a apo CIII desenvolvem hipertrigliceridemia severa, aumento moderado de colesterol e aumento de ácidos graxos livres (Ito *et al.*, 1990,

Aalto-Setala *et al.*, 1992). Este fenótipo resulta principalmente de uma redução na taxa fracional de remoção plasmática das VLDL nestes animais (Aalto-Setala *et al.*, 1992). Mesmo na presença de elevados níveis de TG e AGL, a homeostase glicêmica e secreção de insulina nestes animais são normais (Amaral *et al.*, 2002). Quando submetidos à dieta controle, estes animais aparentemente não diferem dos controles em termos de comportamento geral, crescimento, fertilidade e longevidade. Quando submetidos à dieta rica em colesterol eles desenvolvem mais aterosclerose que os controles (Hayek *et al.*, 1995). Estudos com camundongos *knockout* do gene da apo CIII mostraram maior quantidade de tecido adiposo visceral quando comparados aos *wild-type* na vigência de dieta rica em gordura, porém não apresentaram diferenças no tamanho dos adipócitos (Duivenvoorden *et al.*, 2005). Verificou-se que os animais *knockouts* apresentavam também maior atividade da lipoproteína lípase, portanto, gerando maior disponibilidade de ácidos graxos para serem captados pelo tecido adiposo.

Alberici *et al.* (2003) demonstraram uma maior respiração mitocôndrial de repouso (não fosforilante) no fígado de camundongos que super expressam a apo CIII quando comparados aos seus controles. Este aumento da respiração mitocôndrial de repouso não estava relacionado com maior atividade ou expressão das proteínas desacopladoras (UCPs). Em concordância com estes dados, Alberici *et al.* (2006) observaram uma taxa metabólica corporal aumentada, avaliada pelo aumento da produção de CO_2 e temperatura retal dos animais intactos. Como estes resultados revelam uma maior dissipação de energia nos animais apo CIII seria esperado que os mesmos fossem mais magros ou tivessem uma ingestão alimentar aumentada. De fato, os autores verificaram uma ingestão alimentar significativamente maior em CIII sem correspondente aumento de ganho de peso. Estes resultados demonstram que as hiperlipidemias primárias levam a um aumento na respiração de repouso de mitocôndrias hepática, o que pode representar uma adaptação regulatória para oxidar o excesso dos ácidos graxos nas células dos animais que super expressam a apo CIII.

4- Obesidade e hiperlipidemias

A obesidade pode ser definida clinicamente como peso corporal aumentado devido ao acúmulo de tecido adiposo, de magnitude suficiente para produzir conseqüências adversas à saúde (Spiegelman & Flier, 2001). Segundo dados de 2003 da Organização

Mundial de Saúde (OMS), estima-se que 1 bilhão de pessoas estejam com sobrepeso em todo o mundo, sendo que destas, aproximadamente 300 milhões são consideradas obesas. A atual epidemia global de obesidade reflete profundas alterações nos padrões sociais e comportamentais das comunidades ao longo das últimas décadas. A alta velocidade de urbanização, a elevação da renda *per capita* e a redução da demanda por trabalhos que exigem esforço físico, resultaram em um maior consumo de alimentos industrializados, ricos em gorduras saturadas e açúcares, e ao aumento do sedentarismo (Monteiro *et al.*, 2004; Spiegelman & Flier, 2001).

Nas últimas duas décadas, a incidência de obesidade praticamente triplicou em algumas regiões da América do Norte, Europa e Pacífico (OMS, 2003). Embora em certos países, como a China e o Japão, o número de obesos não ultrapasse 5% da população, nos Estados Unidos e em parte da Europa, cerca de dois terços dos adultos encontram-se acima do peso (James, 2004). A obesidade, no entanto, não está restrita somente a países desenvolvidos, uma vez que o número de indivíduos com excesso de peso vem crescendo também na América Latina, Caribe e Ásia (OMS, 2003; James 2004). No Brasil, dados da Pesquisa Nacional de Saúde e Nutrição (Cercato *et al.*, 2004) indicam que 40% da população adulta apresenta algum grau de sobrepeso, enquanto outros estudos populacionais demonstram que atualmente são encontrados dois casos de obesidade para cada caso de desnutrição entre mulheres, exatamente o inverso do observado há apenas 30 anos atrás (Monteiro *et al.*, 2004).

Diversos estudos demonstram que o excesso de adiposidade está relacionado com hipertrigliceridemia, hipertensão, redução de HDL-colesterol e intolerância à glicose e/ou resistência à insulina (Cercato *et al.*, 2004; Ten & Maclaren, 2004; Isomaa *et al.*, 2001). Essas alterações, quando manifestas conjuntamente, resultam na síndrome pluri-metabólica ou síndrome X, a qual está relacionada diretamente ao aparecimento de aterosclerose prematura e a elevação da morbidade e mortalidade por doenças cardiovasculares (Isomaa *et al*., 2001). Além de apresentarem maior incidência de doenças cardiovasculares, indivíduos obesos também são mais propensos ao desenvolvimento de diabetes, de certos tipos de câncer, de doenças respiratórias, de infertilidade e desordens músculo-esqueléticas (Ten & Maclaren, 2004).

A dislipidemia que ocorre na obesidade é caracterizada pelo aumento de lipoproteínas ricas em triglicérides, tanto no período pós-prandial como no jejum, redução

de HDL e elevação da concentração das partículas de LDL pequenas e densas (Watson *et al.*, 2003). A superprodução hepática de VLDL aparentemente consiste no principal defeito causado pelo estado de resistência à insulina que acompanha a obesidade. A incapacidade de suprimir a produção de glicose pelo fígado e a liberação de ácidos graxos pelo tecido adiposo, devido à resistência periférica à insulina, aumenta o aporte desses dois substratos para o próprio tecido hepático, levando ao aumento da produção de VLDL (Watson *et al.*, 2003). Na obesidade, também devido ao estado de resistência à insulina, ocorre uma diminuição da taxa de degradação da apo B-100, o que contribui para secreção ainda maior de VLDL.

Além do aumento da secreção de VLDL, a resistência à insulina na obesidade provoca também uma redução na remoção das lipoproteínas ricas em TG. Tal efeito se deve à diminuição da atividade da LPL, cuja síntese é estimulada pela insulina (Chan *et al.*, 2004), e também pela menor atividade dos receptores de LDL, contribuindo assim para o atraso na remoção de VLDL do plasma (Espírito Santo *et al.*, 2005).

Nos indivíduos obesos, observam-se também alterações na composição e na distribuição das partículas de LDL. O aumento da produção de VLDL, combinado ao atraso na sua remoção do compartimento plasmático, induz o aumento das trocas entre colesterol esterificado de LDL e triglicérides de VLDL, mediadas pela CETP. Essas trocas produzem partículas de LDL enriquecidas em triglicérides, que são rapidamente lipolizadas no fígado pela lipase hepática gerando partículas mais densas e menores (Watson *et al.*, 2003). As atividades da CETP e da LH também parecem estar aumentadas na obesidade, ocasionando a produção de partículas de VLDL mais ricas em colesterol e potencialmente mais aterogênicas (Chan *et al.*, 2004).

A maior parte das anormalidades no metabolismo de lipoproteínas observadas em indivíduos obesos é decorrente da resistência à insulina, porém não se sabe se a recíproca é verdadeira, isto é, se as hiperlipidemias primárias (genética) podem ter uma relação causal com obesidade.

II - HIPÓTESES DE TRABALHO

Hipótese 1: A super-expressão da CETP aumenta a trigliceridemia a curto prazo (pós-prandial).

Considerando que a CETP é uma proteína que transfere triglicérides das LP ricas em TG (quilomícrons, VLDL e remanescentes) para as HDL, postulamos que o organismo que expressa maior quantidade de CETP apresentaria maiores níveis de TG de origem intestinal na fração HDL e que esta LP, permanecendo mais tempo no compartimento plasmático, elevaria a trigliceridemia pós-prandial. (Figura 1)

Hipótese 2: A super-expressão da CETP reduz a adiposidade a longo prazo.

Considerando que, ao promover a transferência de TG dos quilomícrons para as HDL, a CETP está favorecendo o aumento do aporte de ácidos graxos para o fígado (via HDL-TG) e reduzindo o aporte de ácidos graxos para os tecidos extra-hepáticos (via quilomícrons), em longo prazo, esta redistribuição da fonte de ácidos graxos para os tecidos poderia levar a redução da massa adiposa no organismo que expressa maior quantidade de CETP. (Figura 2).

Hipótese 3: A super-expressão da apolipoproteína CIII reduz a adiposidade.

Considerando que a) a super-expressão de apo CIII prejudica o *clearance* das LP ricas em TG, reduzindo o aporte de ácidos graxos para os tecidos, b) a super-expressão da apo CI, apolipoproteína da mesma família que também causa hipertrigliceridemia, resulta em proteção contra obesidade, c) a deficiência de apo CIII causa hipotrigliceridemia e aumento de adiposidade, e d) a super-expressão de apo CIII provoca o aumento de mecanismo intracelular mitocôndrial de dissipação de energia, postulamos que organismos que expressam maiores quantidades de apo CIII apresentariam menor adiposidade. (Figura 3).

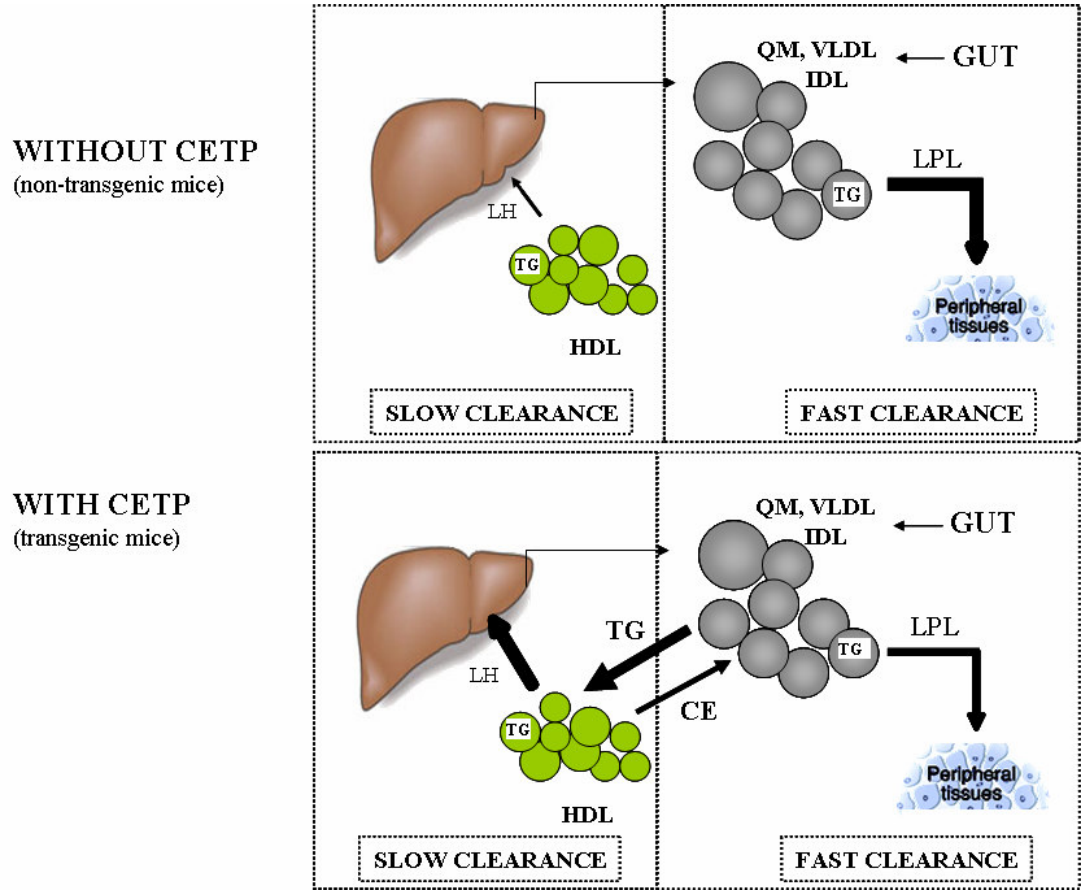

Figura 1. A super-expressão da CETP aumenta a trigliceridemia a curto prazo (pós-prandial) (Hipótese 1). Na parte superior mostramos o fluxo dos TG do plasma na ausência de CETP, como ocorre nos camundongos controles que não expressam a CETP. Os TG das lipoproteínas ricas em triglicérides são rapidamente removidos da circulação porque são hidrolisados pela LPL liberando os AGL para os tecidos extra-hepáticos. Uma pequena parte dos TG plasmáticos é removida mais lentamente pela ação da LH sobre a HDL liberando AGL para o fígado. Na parte inferior mostramos a fluxo dos TG do plasma na presença da CETP, como ocorre nos camundongos transgênicos. Neste modelo ainda ocorre a hidrólise dos TG pelas LPL e rápida captação dos AGL pelos tecidos periféricos. Porém, pela ação da CETP, parte significativa dos TG destas lipoproteínas é transferida para as HDL e através da LH os AGL são liberados para o fígado. Por permanecer mais tempo na circulação, as HDL enriquecidas em TG elevam a trigliceridemia. O redirecionamento do fluxo de TG para as HDL, mediado pela CETP, poderia ser mais bem evidenciado no estado pós-prandial.

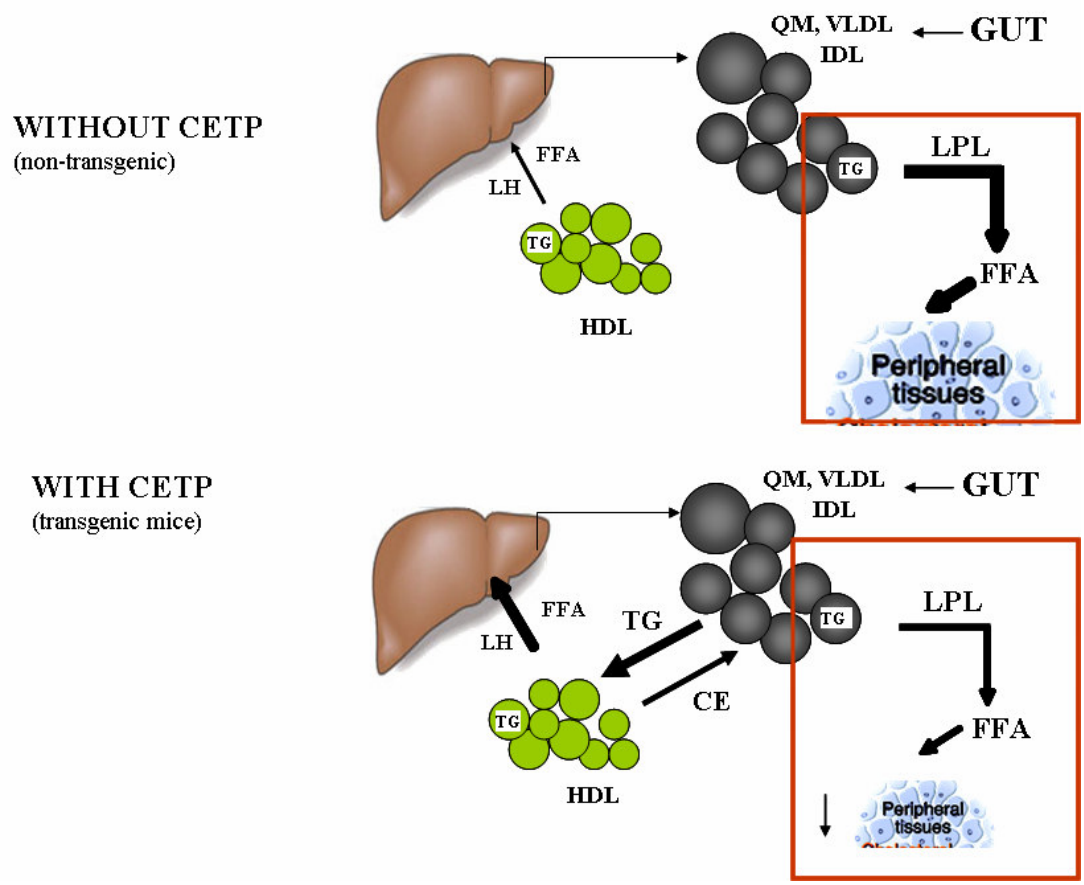

Figura 2. A super-expressão da CETP reduz a adiposidade a longo prazo (Hipótese 2). Na ausência de CETP (parte superior) a grande quantidade de TG circulante vai ser primariamente hidrolisada pela LPL e os ácidos graxos serão captados pelos tecidos periféricos (adiposo). Na presença da CETP (parte inferior) uma quantidade significativa dos TG dos quilomícrons serão transferidos para as HDL. As HDL enriquecidas de TG vão ser hidrolisadas pela LH, liberando os AGL para o fígado. Desta maneira, a CETP desvia parte do fluxo de AGL da periferia (adiposo) para o fígado. Numa situação de excesso de TG da dieta, e a longo prazo, este desvio de ácidos graxos do adiposo para o fígado poderia resultar em menor formação de tecido adiposo no animal que super-expressa a CETP.

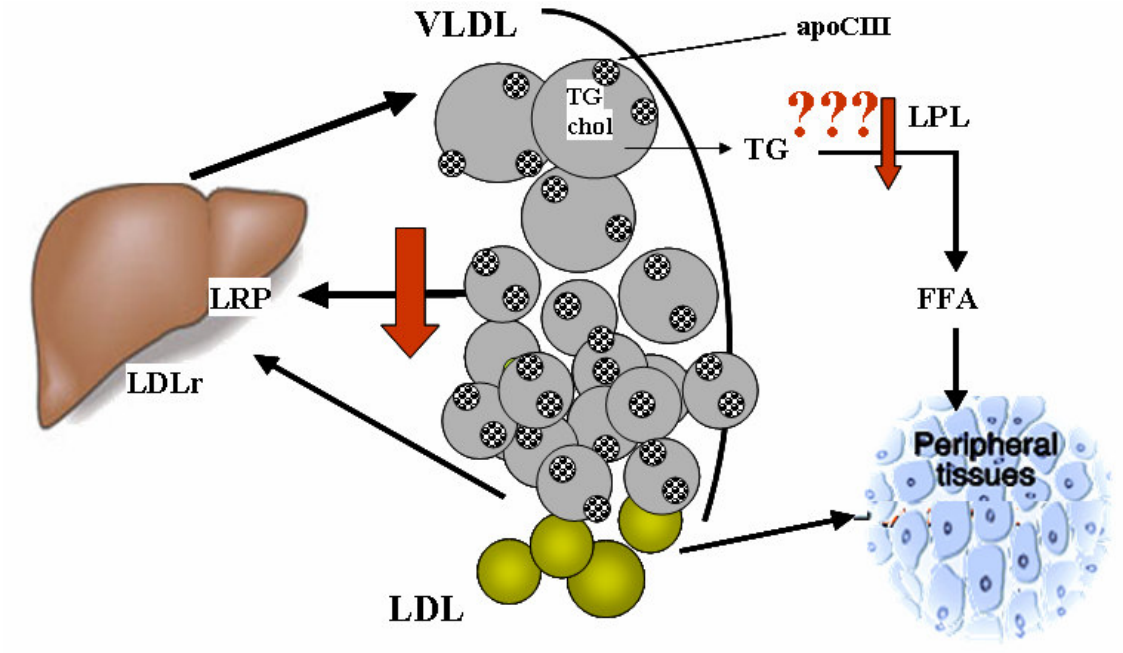

Figura 3. A super-expressão da apolipoproteína CIII reduz a adiposidade (Hipótese 3). As apoCIII são constituintes das lipoproteínas ricas em TG, que podem ser de origem hepática ou intestinal. O aumento da quantidade de apoCIII na superfície das LP ricas em TG prejudica o seu reconhecimento por receptores hepáticos específicos, os LRP. Isso faz com que estas partículas permaneçam mais tempo na circulação sanguínea. O excesso de apoCIII poderia também levar a inibição da LPL resultando em menor geração de AGL que são captados pelo tecido adiposo. A inibição direta da apoCIII sobre a LPL ainda é questionada na literatura (Katrina Aalto-Settala et al. em 1992 e 1996). De qualquer maneira, o prejuízo do *clearance* das LP ricas em TG na presença de grandes quantidades de apoCIII resulta em hipertrigliceridemia e possivelmente em redução do fluxo de ácidos graxos livres para o tecido adiposo diminuindo sua formação.

III - OBJETIVOS

- **OBJETIVO GERAL**

Investigar o efeito da expressão da CETP e da apolipoproteína CIII sobre o metabolismo de triglicérides provenientes da dieta a curto e longo prazo, em camundongos transgênicos e controles.

- **OBJETIVOS ESPECÍFICOS**

1- Investigar o efeito da expressão da CETP na trigliceridemia pós-prandial, bem como mecanismos envolvidos nestes efeitos;
2- Investigar o efeito da expressão da CETP e da apolipoproteína CIII, isoladas ou combinadas, sobre a adiposidade induzida por dieta rica em gordura.

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V. 1. Plasma CETP activity increases postprandial triglyceridemia due to delayed plasma clearance of triglyceride rich lipoproteins: studies in CETP transgenic mice

Plasma CETP activity increases postprandial triglyceridemia due to delayed plasma clearance of triglyceride rich lipoproteins: studies in CETP transgenic mice.

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Running Title: CETP retards triglyceride plasma clearance

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ABSTRACT

The effect of CETP levels on the postprandial intravascular metabolism of triglycerides (TG) is an often-overlooked aspect of the relationship between CETP and lipoprotein (LP) metabolism**.** In this work, we hypothesized that, by transferring TG to HDL, CETP delays plasma clearance of TG rich LP, what would be best observed in the postprandial state. Thus, the oral fat tolerance, fat absorption, VLDL secretion and TG rich lipoprotein plasma clearance were compared in human CETP expressing transgenic (Tg) and CETP non-expressing control (Non-Tg) mice. The postprandial triglyceridemia curve was markedly increased in CETP-Tg mice compared to controls (280 ± 30 vs. 190 ± 20 mg/dL.6h, respectively, p= 0.02). No differences in intestinal fat absorption and VLDL secretion rates were observed between both groups of mice. Kinetic studies of double labeled chylomicron-like emulsions showed that both [^{3}H] triolein and [^{14}C] cholesteryl oleate fractional clearance rates were significantly slower (~20%) in CETP Tg than in Non-Tg mice. These results may be explained by a 50% reduction in post-heparin plasma lipoprotein lipase activity verified in CETP Tg mice. Thus, we provide direct evidences that plasma CETP activity impairs the postprandial metabolism of triglyceride rich lipoproteins**.** These findings contribute to the understanding of the influence of specific genes on the lipoprotein responsiveness to dietary fat and may help in the identification of individuals more sensitive to high-fat diets.

Key words: CETP, triglycerides, lipoprotein lipase, dietary fat, postprandial lipoproteins

INTRODUCTION

Postprandial lipemia is a physiological phenomenon occurring several times a day to cope with the almost complete absorption of dietary fat (Karpe 2002). It is characterized by the transient accumulation in plasma of potentially atherogenic particles of intestinal and hepatic origin, named chylomicrons, VLDL, and their remnants. The potential atherogenic role of postprandial triglyceride (TG) rich lipoproteins was first hypothesized by Zilversmit (1979). These lipoproteins, particularly small chylomicrons and VLDL remnants, may infiltrate and undergo retention in the vessel wall (Patsch et al. 1992; Chung et al. 1994; Karpe, 1999; Welty et al. 1999).

The reasons for the relative lack of clinical evidence for the involvement of postprandial lipoprotein metabolism in the development of atherosclerosis are probably consequences of biological, statistical and methodological issues (Karpe, 1999). However, several studies have shown that TG rich lipoproteins are coronary risk factors, independently of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels (Kawakami & Yoshida 2005, Fruchart et al. 2004, Karpe et al. 2001; McNamara et al. 2001).

Cholesteryl ester transfer protein (CETP) takes part in the intravascular metabolism of TG rich lipoproteins as well as of HDL. Several studies have shown that the induction of postprandial lipemia is accompanied by an increase in plasma CETP activity (Tall et al. 1986; Castro & Fielding 1985; Contacos et al. 1998, Lottenberg et al., 1996). This mainly reflects the higher levels of substrates for the CETP mediated reaction, which transfers cholesteryl ester from HDL to the apolipoprotein B containing lipoproteins in exchange for TG. Thus, CETP transforms cholesteryl ester-enriched HDL into cholesterol-poor, TG enriched HDL, especially in the postprandial state (Tall et al. 1986, Guerin et al. 2002). By

reducing HDL cholesterol levels, CETP has been considered a major target for developing inhibitors aimed at reducing atherosclerosis risk (for review see Clark, 2006, Milani & Lavie, 2006). However, recent drawbacks in clinical trials have seriously questioned this possibility (Pearson, 2006, Pfizer News, 2006).

Although the rise in plasma CETP activity in the fed state is well characterized, results from animal and human studies have not agreed on the variation of CETP mass in response to feeding or to a fat meal load. MacLean et al (2000) reported that plasma CETP concentration increased in fed compared to fasted CETP transgenic mice. In contrast, Jiang et al. (1991) showed that CETP expression levels in fasted hamsters fell after feeding. A fat load after an overnight fast did not change (Lottenberg et al. 1996) or increased (Noone et al., 2000) the plasma CETP concentration in normolipidemic subjects.

While the consequences of the postprandial lipemia on the CETP levels and cholesterol fluxes between lipoproteins have been substantially studied, the reciprocal effect of the CETP levels on the postprandial intravascular metabolism of TG is an often-overlooked aspect of the relationship between CETP and lipoprotein metabolism. In this work, we hypothesized that, by transferring TG to HDL, CETP delays plasma clearance of TG rich lipoproteins, what would be best observed in the postprandial state. Thus, we compared human CETP expressing transgenic with CETP non-expressing control mice in terms of fat tolerance, fat absorption, VLDL secretion and TG rich lipoprotein plasma clearance.

MATERIAL AND METHODS

Animals

All animal protocols were approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP). Hemizygous human CETP transgenic mice (line 5203, C57BL6/J background) (Jiang et al. 1992) expressing a human CETP minigene under the control of its natural flanking sequences were derived from Dr. Alan R. Tall's colony (Columbia University, New York, NY). Crosses of CETP transgenic and C57BL6 mice from the university's animal care center (CEMIB/UNICAMP) have been maintained for 10 years. CETP transgenic mice and non-transgenic mice (Non-Tg) littermates, between 12 and 16 weeks of age were used in the present study. Only female mice were used for the experiments. Mice were housed in a temperature-controlled room on a 12 h light-dark cycle and they had free access to water and food and were placed on a standard chow diet (Nuvital CR1, Colombo, Brazil). CETP-expressing mice were genotyped by assaying the plasma CETP activity (Berti et al. 2001).

Plasma biochemical analyses

Blood samples were obtained by either the tail or the retro-orbital plexus of fasted anesthetized mice. Plasma cholesterol (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany), triglycerides (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany), free fatty acids (Wako Chemical, Neuss, Germany) and glucose (Glucose GOD PAP-Laborlab, SP, Brazil) were determined using enzymatic colorimetric assays according to the manufacturer's instructions.

Plasma cholesteryl ester transfer protein activity assay

The CETP-mediated transfer of cholesteryl ester was determined as previously described (Berti et al. 2001). Briefly, a mixture of acceptor lipoproteins (human VLDL and LDL) was

incubated with human HDL_3 labeled with [^{14}C]-cholesteryl oleate (Oliveira & Quintão, 1996) and 10 µl of mouse plasma as the source of CETP in a final volume of 100 µl. Blanks were prepared with Tris/saline/EDTA buffer (10 mM/140 mM/1 mM), pH 7.4, and negative controls with plasma from non-transgenic mice. After incubation at 40 ˚C for 4h, the apoB containing lipoproteins were precipitated with a solution of 1.6% dextran sulfate/1 M $MgCl_2$ (1:1) and the radioactivity was measured in the supernatant using a LS6000 Beckman Beta Counter.

Mice fat tolerance test

Mice fasted for 12 hours received through a gastric tube a bolus of soybean oil (13 mL/Kg of body weight). Blood samples were obtained by the tail tip at 0 (just before the fat load) and 60, 120, 180, 240, 300 and 360 min (Ebara et al. 2000). Plasma triglyceride levels were measured as described above. The baseline triglyceride level was subtracted from the postprandial levels at each time point and the incremental areas under the TG curves were calculated using the program Microcal™ Origin™ 4.1.

Dietary fat absorption

Mice that were housed in individual cages had their food ingestion weighed and stools collected for 72 hours. Fat content from dried and powdered food pellets and stools were measured by weighing the dried lipid extracts (Folch, 1957). Dietary fat absorption was estimated as the difference between the fat content in ingested food and excreted in stools. Experiments were performed with two types of diets, low fat chow diet (4% fat) and a high fat diet (14% fat). Mice were allowed to adapt to the high fat diet during one week before the 3 day fat balance.

***In vivo* hepatic VLDL secretion rate**

After a 12 h-fasting period or 5 hours after an oral fat load, anesthetized mice (ketamine 50

mg/kg, Parke-Davis, SP, Brazil and xylazine,10 mg/kg, Bayer S.A., SP, Brazil) were injected intravenously with Triton WR1339 (500 mg/Kg body weight) using a 15% (wt/vol) Triton solution in 0.9% NaCl (Otway & Robinson et al. 1967). At 0, 15, 30, 60, 90 and 120 min after the Triton injection, blood samples were drawn and analyzed for cholesterol and triglyceride concentrations. Hepatic VLDL secretion rates were calculated from the slopes of the linear regression curves using the program Microcal™ Origin™ 4.1.

Plasma kinetics of labeled triglyceride rich emulsion

The triglyceride rich lipid emulsions were prepared by sonication and purified by ultracentrifugation as previously described (Oliveira et al., 1988). A lipid mixture containing 23% phosphatidylcholine (Lipids Products, Surrey,U.K.) , 2% cholesterol, 6% cholesteryl oleato and 69% triolein (Nu-Chek Prep., Elysian, MN), 13 µCi of [^{3}H] triolein and 11 µCi [^{14}C] cholesteryl oleate (Amershan Biosciences UK Limited) was evaporated under nitrogen and desiccated under vacuum at 4° C during overnight to eliminate residual solvent. Lipids were emulsified by sonication in 2.785 M NaCl solution at 55 °C, using a 1 cm probe with continuous (30 min.) output of 70-80 W. The triglyceride rich emulsion was purified in two steps of ultracentrifugation in discontinuous gradients of NaCl solutions (densities of 1.065, 1.020 and 1.006 g/mL) in a SW 41 rotor at 22 ° C. Initially, the coarse lipid floating after 12000 rpm for 15 min was discarded and the volume replaced. The emulsion particles floating after a second run at 36000 rpm for 25 min were aspirated and used for the kinetic studies. The lipid emulsion was injected intravenously (femoral) into anesthetized female mice after a 12 h-fasting period (1 x 10^{6} dpm, 0.5 mg TG). Blood samples of 50 µL were taken from the tail tip at 1, 2, 3, 5, and 10 min. Mice were killed by exsanguination through the retro-orbital plexus. Blood samples were centrifuged at 2500 x *g* at 4°C for 10 min. Aliquots of plasma were stored at -80°C until analysis. Neutral lipid

classes were separated by TLC in the solvent system of hexane/diethyl ether/ acetic acid (70:30:1, by vol.). Bands corresponding to triglycerides and cholesteryl ester were then scraped into counting vials for radioactivity measurement in 3 mL of scintillation solution (ACS® Aqueous Counting Scintillant, Amersham Biosciences UK Limited, England) in a Beckman LS 6000 Beta Counter. Plasma fractional clearance rates (FCR) of ^{3}H-triolein and ^{14}C-cholesteryl oleate were computed from monoexponential curve fitting using the program Microcal™ Origin™ 4.1.

Intravascular lipases activities

Total lipase activity was determined according to Ehnholm & Kuusi (1986). Overnight fasted mice plasmas, obtained before and 10 minutes after subcutaneous injection of heparin (100 U/Kg body weight), were incubated with a ^{3}H-triolein/arabic-gum substrate ([9,10 ^{3}H (N)]-triolein, New England Nuclear, Boston, MA) during 1 hour. Hepatic lipase (HL) activity was determined in tubes where the lipoprotein lipase (LPL) was inhibited by 2 M NaCl. The hydrolyzed labeled free fatty acids were extracted with methanol / chloroform / heptane (1.4: 1.25: 1), dried under N_2, and their radioactivity was determined in a LS6000 Beckman Beta Counter. The LPL activity was calculated as the difference between the total lipase and the hepatic lipase activities.

RESULTS

Plasma lipids and glucose

Table 1 shows plasma lipid and glucose levels in female CETP Tg and Non-Tg littermates mice. They exhibited the expected lipemic phenotype, i. e., plasma total cholesterol levels were reduced by 20% in CETP Tg mice compared to the Non-Tg mice. No differences in glucose, TG and FFA levels were found between the two groups.

Fat tolerance test

We evaluated the overall intravascular metabolism of gut-derived particles by assessing the extent of postprandial lipemia after an oral fat load in CETP Tg and Non-Tg mice. The large increase in postprandial TG peaked at 2 hours after the fat load in both groups **(Figure 1)**. The integrated 6-hour incremental area under the triglyceridemia curve (above the basal levels of TG) was markedly increased in CETP-Tg mice than in their controls (280 ± 30 vs. 190 ± 20 mg/dL.6h, respectively, p= 0.02). A time point-by-point comparison of the curves shows statistical differences at 2 and 5 hours after the fat load. The prolonged residence time of TG in the plasma compartment of the CETP Tg mice could be the result of i*)* increased intestinal absorption, *ii)* increased hepatic VLDL-TG secretion, and *iii)* decreased clearance of TG from the circulation.

Fat absorption

A three-day fat balance (ingestion - excretion) was performed in both groups of mice in order to estimate the dietary fat absorption. The results presented in **Table 2** show that there are no differences in the intestinal absorption of dietary fat in both groups of mice under low fat chow diet (4 % fat). When mice were submitted to a high fat diet (14 % fat), we observed that Non-Tg mice absorbed larger amounts of dietary fat than CETP Tg because they actually ate more food and not because they had greater intestinal fat

absorption capacity. Thus, higher postprandial plasma levels of TG observed in CETP Tg mice (Figure 1) can not be attributed to increases in the capacity of intestinal fat absorption.

Hepatic VLDL secretion rates

Because triglyceride levels were still significantly elevated 5 hours after the oral fat load in CETP Tg mice (Figure 1), we evaluate the effect of CETP on the hepatic triglyceride secretion rate in Triton treated mice. The accumulation of VLDL-TG in the plasma was monitored along time in overnight fasted mice **(Figure 2 A)** and 5 hours after the oral fat load **(Figure 2 B)**. The triglyceride accumulation rates were calculated from the slopes of the curves (linear regression) and no differences were observed between CETP and Non-Tg mice either in the fasted mice (3.2 ± 0.6 vs. 3.1 ± 0.6 mg/dL/h) and 5 hours after the oral fat load (11 ± 1.2 vs. 12 ± 1.6 mg/dL/h). Likewise, cholesterol accumulation rates were not affected by the presence of CETP in mice in both conditions, fasting and post fat load (data not shown).

In vivo plasma clearance of TG-rich chylomicron-like emulsion particles

Others and we have previously shown that these protein free lipid emulsions are models to study native chylomicron metabolism (Redgrave & Maranhão, 1985; Hirata et al, 1987; Oliveira et al., 1988; Zerbinatti et al, 1991). These emulsions promptly acquire plasma apolipoproteins and are readily hydrolyzed by lipoprotein lipase. The plasma removal of the emulsion double labeled with [^{3}H] triolein and [^{14}C] cholesteryl oleate injected intravenously into CETP Tg and Non-Tg mice are shown in **Figure 3.** The plasma fractional clearance rate (FCR) of [^{3}H]-triolein was significantly slower in CETP Tg than in control mice (0.157 ± 0.010 vs. 0.187 ± 0.009, respectively, $p < 0.04$)**.** The [^{14}C]-cholesteryl oleate FCR was also slower in CETP Tg than in controls (0.105 ± 0.003 vs. 0.128 ± 0.006, respectively, $p < 0.004$). In a system without CETP, the cholesteryl ester (CE) moiety of the

emulsion is a marker of the remnant particle removal. However, in the presence of CETP, there may be considerable dilution of the radioactive CE in the emulsion with cold CE coming from endogenous HDL, decreasing its specific activity as compared to the emulsion in the plasma compartment of Non-Tg mice. This process may falsely indicate a decrease in [^{14}C]CE-FCR in CETP Tg. On the other hand, plasma removal rate of [^{3}H]TG is not subjected to any artifact and results from the rate of several process, i.e., degradation by LPL, remnant tissue uptake and, in the case of CETP Tg mice, CETP mediated transfer to HDL, which has a longer half-life in the plasma compartment.

Plasma Lipoprotein lipase activity

In order to investigate the involvement of intravascular lipolysis in the delay of [^{3}H]TG-FCR we measured the maximal plasma activity of lipoprotein lipase in post-heparin plasma from both groups of mice. We found a 50% decrease in LPL activity in CETP Tg as compared to Non-Tg (228 ± 19 vs. 466 ± 46 nmol/mL/h, respectively, $p<0.001$) and no significant differences in hepatic lipase activity were found (887 ± 83 vs. 1041 ± 101 nmol/mL/h, respectively).

DISCUSSION

Although genes coding for some apolipoproteins and LPL are well characterized as determinants or potential markers for postprandial lipemic responses, studies implicating CETP in this process are lacking. In this work, we performed functional studies designed to show direct evidences that plasma CETP activity modifies postprandial response of triglyceride rich lipoproteins. This was done by comparing a biological context where CETP levels varied as all-or-nothing in human CETP transgenic mice as compared to the wild type mice. We demonstrated that high levels of plasma CETP activity are associated with dietary fat intolerance.

Interestingly, the TaqI polymorphism of the human gene encoding CETP that is associated with low HDL cholesterol and high CETP activity (B1 allele) has also higher plasma triglyceride concentrations (Ye & Kwiterovich, 2000). In addition, simvastatin treatment of normotriglyceridemic patients with coronary heart disease decreased CETP activity dose-dependently and improved the TG response to an oral fat loading test (van Wijk et al., 2005). Although the improvement in the postprandial TG plasma removal is certainly dependent on the upregulation of LDL receptors promoted by simvastatin, our data indicate that lower CETP plasma levels may also have contributed this effect.

Contrary to our results, Ritsch et al. (1997) reported one case of a CETP deficient patient with very high postprandial plasma levels of TG and impaired TG tolerance after an oral fat meal. However, in this case the CETP mutant exhibited an apoE3/E2 phenotype, which is likely the main cause of the TG intolerance.

Possible mechanisms involved in these CETP effects were further investigated in mice. Differences in intestinal fat absorption and in hepatic triglyceride secretion rate were ruled out. Nonetheless, kinetic studies with labeled chylomicron like emulsions showed that

the lack of CETP expression accelerates TG rich lipoproteins plasma removal rates by about 20%. The reasons for the CETP mediated delay in TG plasma clearance can be explained by a decreased LPL mediated lipolysis, since we showed lower maximal in vitro LPL activity in CETP Tg mice plasma. However, we can not totally exclude a possible reduction in receptor mediated remnant tissue uptake. It was already previously demonstrated that CETP expressing mice present a reduction in LDL receptor expression (Jiang et al., 1993). However, to our knowledge, there are no reports on the CETP effects on the expression of the most important remnant receptor, the LRP.

The mechanisms underlying the differential lipemic responses of CETP expressing and non-expressing mice could also be true for humans with high and low CETP activities, but direct confirmation with additional studies are needed.

Our findings disclose a new aspect related to the effects of the CETP expression and contribute to an improved understanding of the influence of specific genes on the lipoprotein responsiveness to dietary fat. Furthermore, these results may help in the identification and counseling of individuals more sensitive to high-fat diets.

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FIGURE LEGENDS

Figure 1 - Oral fat tolerance test in CETP transgenic (Tg) and non-transgenic (Non-Tg) mice. Incremental areas under the triglyceridemia curves are: 280 ± 30 and 190 ± 20 (mg/dL.6h) for CETP and Non-Tg, respectively; p= 0.02 by Student t test. Mean ± SE (n=9-13). * $p<0.05$ by Student t test.

Figure 2 - VLDL-triglyceride secretion in CETP transgenic (Tg) and non-transgenic (Non-Tg) mice after an overnight fasting (A) or five hours after a fat load (B). Mice were injected with Triton WR1339 as described in Material and Methods Sections. Mean ± SE (n=7-9). Slopes of the triglyceridemia curves are: A) 3.2 ± 0.6 and 3.1 ± 0.6 mg/dL/h and B) 11 ± 4 and 12 ± 3 mg/dL/h, for CETP and Non-Tg mice, respectively.

Figure 3 - Plasma clearance of chylomicron like emulsions double labeled with ^{3}H-triolein (A) and ^{14}C-cholesteryl ester (B) injected intravenously in CETP transgenic (Tg) and non-transgenic (Non-Tg) mice. Plasma fractional clearance rates are: A) 0.157 ± 0.010 and 0.187 ± 0.009 (n= 8, $p<0.04$) and B) 0.105 ± 0.003 and 0.128 ± 0.006 (n= 8, $p<0.004$), for CETP and Non-Tg mice, respectively (Student t test).

Table 1. Fasting plasma concentration of lipids and glucose in female mice expressing CETP and control non transgenic mice.

	CETP	Non-Tg
GLUCOSE (mg/dL)	92 ± 5	90 ± 5
CHOL (mg/dL)	56 ± 2 *	71 ± 6
TG (mg/dL)	73 ± 5	80 ± 3
FFA (mmol/L)	0.5 ± 0.1	0.5 ± 0.1

CHOL: Cholesterol, TG: Triglycerides, FFA: free fatty acid.
Mean ± SEM. (n=7-14). * $p < 0.05$.

Table 2. Intestinal absorption* of dietary fat in female mice expressing CETP and control non transgenic mice on a low or high fat diets.

	Low Fat Diet			High Fat Diet		
	Fat ingestion	Fat absorption		Fat ingestion	Fat absorption	
	(mg)	(mg)	%	(mg)	(mg)	%
Non-Tg	510 ± 29.1	327 ± 35.1	64 ± 4	1546 ± 31.8 [a]	1485 ± 36.6 [b]	96 ± 1
CETP	512 ± 12.6	302 ± 16.0	59 ± 3	1356 ± 56.4 [a]	1304 ± 61.9 [b]	96 ± 1

*Estimated as the difference between food fat ingestion and fat excretion in stools during 72 hours. Low fat chow diet: 4% fat, and high fat diet: 14% fat. The same letters indicate the pair comparisons that are significantly different: a,b: $p<0.05$ (Student t test). Mean ± SE (n=6).

Figure 1

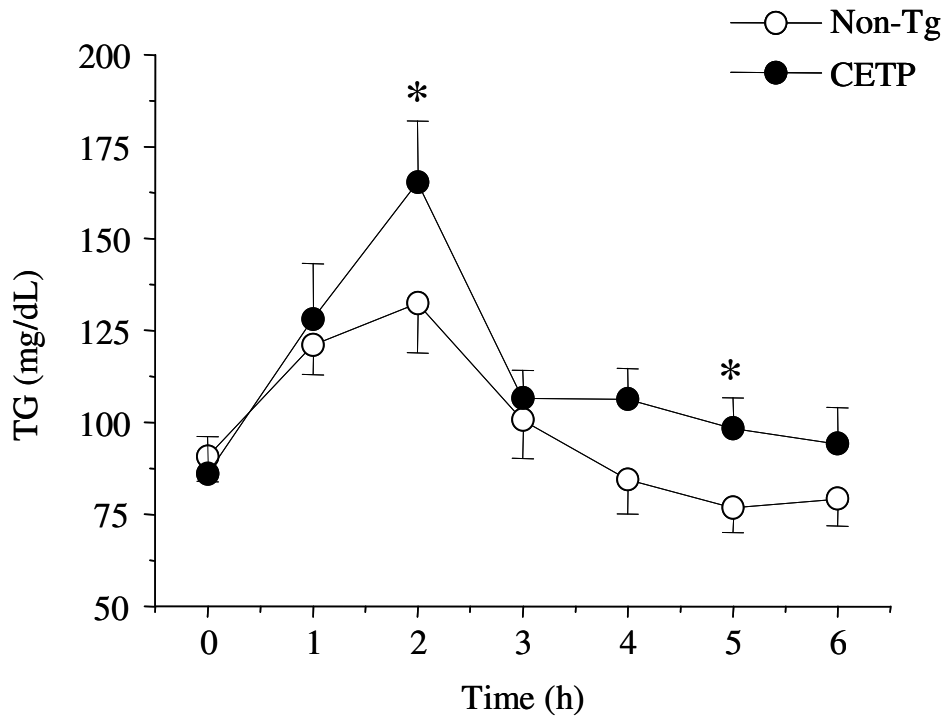
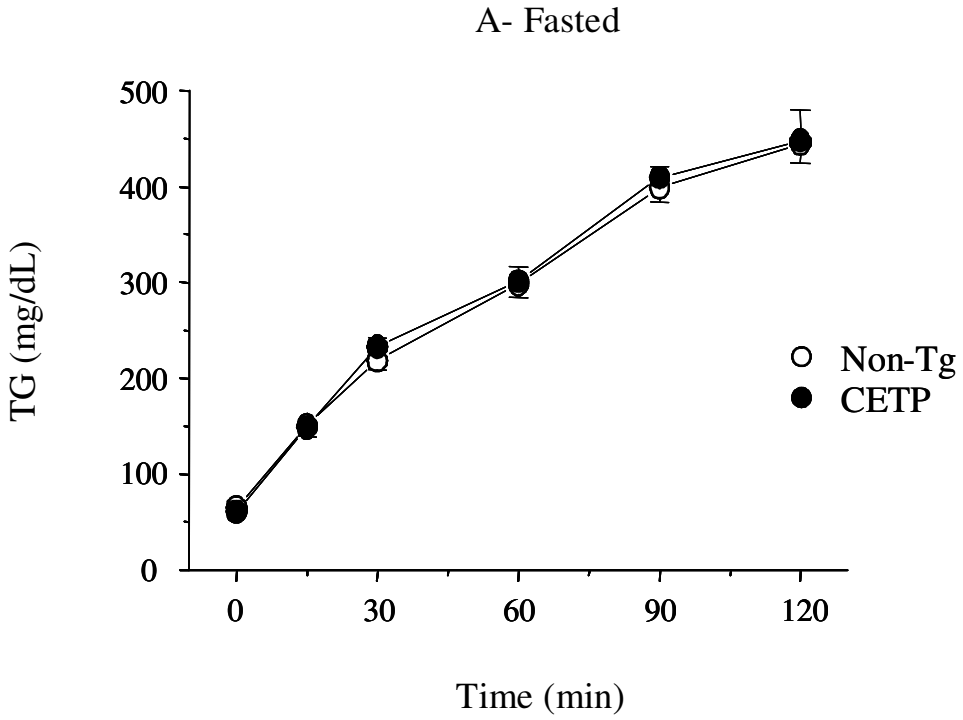


Figure 2


A- Fasted
500
400
300
200
100
0
TG (mg/dL)
0
30
60
90
120
Time (min)
Non-Tg
CETP

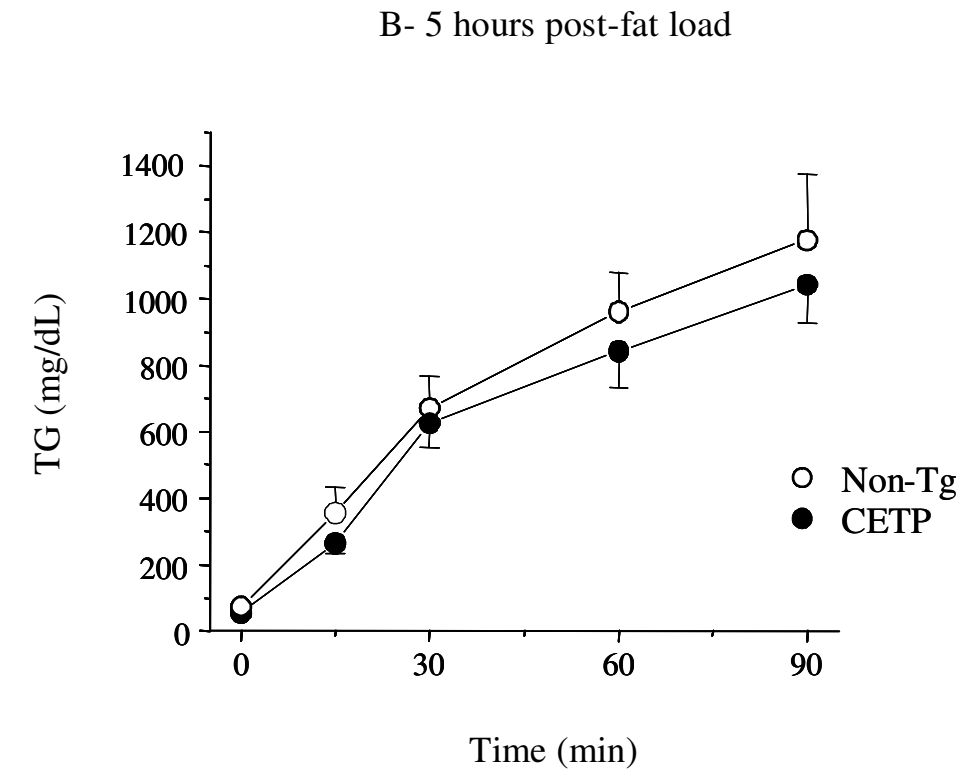
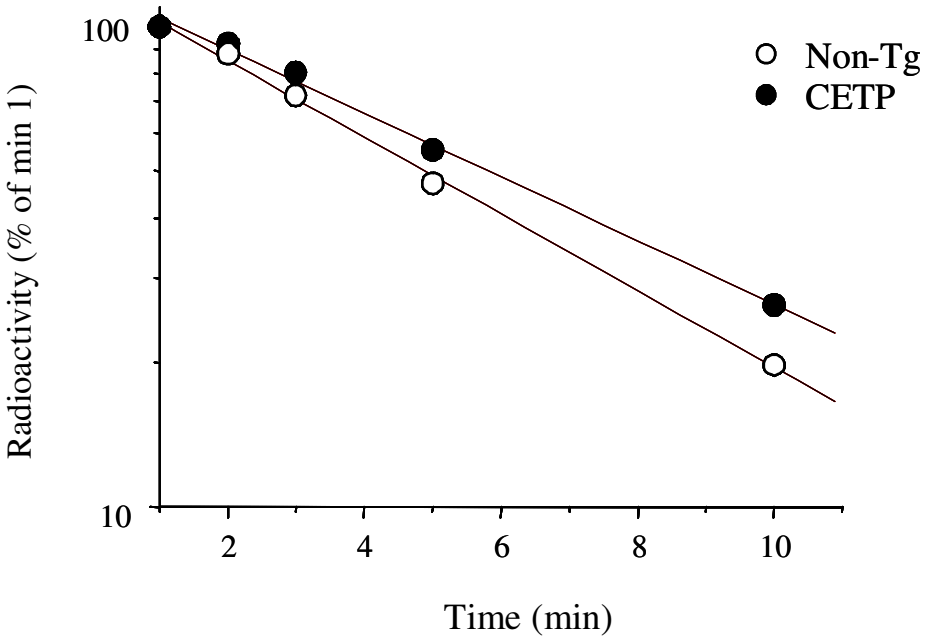
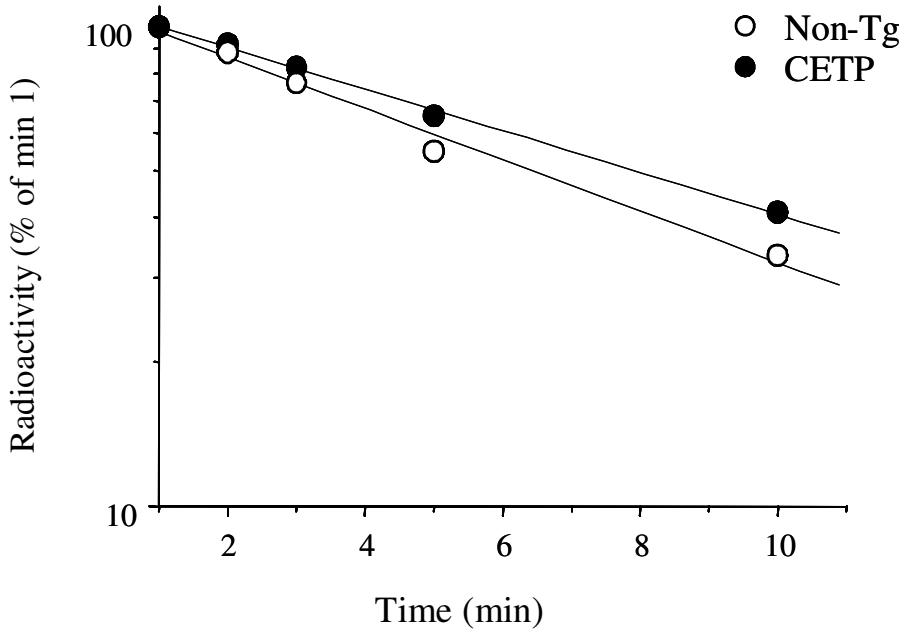

B- 5 hours post-fat load
1400
1200
1000
800
600
400
200
0
TG (mg/dL)
0
30
60
90
Time (min)
Non-Tg
CETP

Figure 3

A: ^{3}H - Triolein



B: ^{14}C – Cholesteryl Oleate



V. 2. Overexpression of apolipoprotein CIII increases and CETP reverses diet induced obesity in transgenic mice

Overexpression of apolipoprotein CIII increases and CETP reverses diet induced obesity in transgenic mice

Running Title: Apolipoprotein CIII increases and CETP reduces obesity

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ABSTRACT

Objective: We recently described that hypertriglyceridemic apolipoprotein (apo) CIII transgenic mice show increased whole body metabolic rate. In this study, we used these apo CIII expressing mice, combined or not with the expression of the natural promoter driven CETP gene, to test the hypothesis that both proteins modulate diet induced obesity.

Measurements and Results: Mice expressing apo CIII, CIII/CETP, CETP and non transgenic (NonTg) mice were maintained on a high fat diet (14% fat by weight) during 20 weeks after weaning. At the end of this period, all groups exhibited the expected lipemic phenotype. Fasting glucose levels were neither affected by the high fat diet nor by the distinct genotypes. However, apo CIII mice showed significantly higher glycemia (~35%) and lower insulin levels (~45%) in the fed state, compared to the NonTg mice. The apo CIII mice presented significantly increased body weight, lipid content of the carcass (~25%), visceral adipose tissue mass (about 2-fold) and adipocyte size (~25%) compared to the CETP and NonTg mice. The CETP expression in the apo CIII background normalized the subcutaneous adipose depot and visceral adipocyte size to the levels of NonTg mice. Plasma leptin levels were lower in CETP groups (25-50%) and higher in the apo CIII mice. Similar core body temperature in all groups and similar liver mitochondrial resting respiration rates in CIII and NonTg mice indicate no differences in basal energy expenditure rates among these high fat fed mice.

Conclusion: The elevation of plasma apo CIII levels aggravates diet induced obesity and the expression of physiological levels of circulating CETP reverses this adipogenic effect, indicating a novel role for CETP in modulating adiposity.

Keywords: CETP, apolipoprotein CIII, diet induced obesity, hypertriglyceridemia, adiposity, leptin, mitochondrial respiration.

INTRODUCTION

Hypertriglyceridemia is a common feature in the general population. Although it can be caused by many factors, including dietary habits, alcohol intake, medication, and different diseases, it is clear that a large number of individuals have a genetic tendency to hypertriglyceridemia (1,2).

Apolipoprotein CIII (apo CIII) is an 8.8 kDa plasma glycoprotein constituent of the triglyceride (TG) rich lipoproteins, synthesized mainly by the liver and to a lesser extent by the intestine (3-7). Apo CIII plays an important role in regulating plasma TG metabolism (4,5,8-10). Transgenic mice expressing human apo CIII have elevated TG levels due to the presence in plasma of enlarged triglyceride-rich lipoproteins with increased apo CIII and decreased apo E compared to controls (11-13). Elevation in plasma TG is proportional to the level of apo CIII gene expression and to the amount of human apo CIII in plasma (14,15). On the other hand, murine apo CIII gene deletion results in hypotriglyceridemia (16). Apo CIII delays the clearance of TG-rich lipoproteins by two ways: i) decreasing the affinity of TG-rich lipoproteins for glycosaminoglycan-bound lipases and biochemical inhibition of lipases (17), and ii) reducing the receptor - mediated uptake of lipolysed VLDL and chylomicron remnants (12,18-21).

Cholesteryl ester transfer protein (CETP) is a plasma protein that promotes the heteroexchange of neutral lipids between circulating lipoproteins leading to the net mass transfer of cholesteryl ester from HDL to the apolipoprotein B-containing lipoproteins (chylomicron, VLDL, and LDL) (22,23). Whereas mice normally lack CETP, humans and rabbits express moderate to high levels of this protein (24). In humans, plasma CETP is synthesized by the liver, spleen, and adipose tissue, with lower levels of expression in small intestine, adrenal, kidney, and heart (23). The tissue showing the most conserved

expression in different species is probably the adipose tissue (25). Transgenic mice expressing human (26,27) or simian (28) CETP exhibit a marked reduction in HDL cholesterol, and increased diet induced atherosclerosis (28, 29). In addition, CETP expression stimulates the selective uptake of HDL-derived cholesteryl ester by the liver and other tissues (30) and by human adipocytes (31,32) and protects against atherosclerosis in specific conditions such as hypertriglyceridemia (33) and sex hormone deficiency (34,35).

The expression of apolipoprotein C and CETP may also have an impact on adiposity. Recently, Zhou et al. (36) showed that adipose tissue specific CETP transgenic mice had adipocytes significantly smaller than those of wild type mice. Regarding the apo CIII, we showed recently that transgenic mice overexpressing apo CIII have higher mitochondrial resting respiration rates and whole body oxidative metabolism which allow these mice to keep body mass similar to the controls in spite of their severe hypertriglyceridemia and elevated food ingestion (37). On the other hand, apo CIII deficiency in knockout mice aggravates diet induced obesity (38), probably through the increased intravascular lipolysis of the TG rich lipoproteins.

In this work, we tested the hypothesis that apo CIII overexpressing mice would be less prone to develop diet induced obesity because i) these mice have impaired plasma triglyceride clearance, ii) increased whole body metabolic rate, iii) apo CIII deficiency leads to augmented adiposity, and iv) overexpression of another apo C family member (apo CI) protected against a genetic type of obesity (39). In addition, we hypothesized that CETP overexpression, perhaps through redistributing TG to HDL, in a long term, could decrease adipose tissue accumulation. Thus, co-expression of both proteins, apo CIII and CETP, could result in additive effects of reducing diet induced adipose tissue formation.

MATERIAL AND METHODS

Animals and diets

All animal protocols were approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP). Human apo CIII transgenic mice (line 3707) (40) were crossbred with human natural promoter driven CETP transgenic mice (line 5203) (27) to obtain CIII, CIII/CETP, CETP and non-transgenic (NonTg) littermates used in these studies. The mice founders were kindly provided by Dr. Alan R. Tall (Columbia University, New York, NY). Heterozygous crossbreeding of apo CIII and CETP transgenic colonies has been kept for ten years using C57Bl6 mice, an obesity prone strain of mice. The mice were housed in a temperature controlled room at 22 ± 1 ˚C on a 12 h light/dark cycle and were fed a high fat diet during 20 weeks from weaning. This diet contained: 21% protein, 59% carbohydrates and 14% saturated (lard) fat by weight, in a total of 446 Kcal/100g, containing AIN93 mineral and vitamin mixtures (see detailed composition below). This diet contained also 0.01% cholesterol that was present in lard. Both male and female mice were used for the experiments. CETP expressing mice were genotyped by assaying plasma CETP activity (41) and apo CIII transgenic had plasma triglycerides levels above 300 mg/dL while nonTg TG levels were below 100 mg/dL.

High fat diet composition (g/Kg): 195 casein, 3 L-cistina, 50 corn starch, 100 maltodextrin, 411 sucrose, 50 cellulose, 130 lard, 10 soy bean oil, 35 mineral mix AIN93GMX, 4 calcium carbonate, 10 vitamin mix AIN93G, 2 choline bitartrate, 0.04 butyl hydroxytolueno (BHT).

Plasma lipids, glucose, insulin and leptin levels

Blood samples were obtained by the tail tip or from the retro-orbital plexus of anesthetized mice. Plasma cholesterol (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany),

triglycerides (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany), and free fatty acids (Wako Chemical, Neuss, Germany) were determined after an overnight fasting using enzymatic colorimetric assays according to the manufacturer's instructions. Ten µl of whole blood precipitated with 5% trichloroacetic acid was used for glucose analysis using the glucose oxidase method (Glucose GOD PAP-Laborlab, SP, Brazil). Non-fasting plasma leptin concentrations were determined by ELISA (Crystal Chem Inc., Chicago, USA) and insulin levels measured by radioimmunoassay (RIA), as previously described (42) using rat insulin standards.

Carcass chemical composition

Fed mouse carcasses (wet weight) were dehydrated to a constant weight at 65°C during 72 hours (dry weight). Total water content was calculated as wet weight minus dry weight. Total carcass fat (subcutaneous adipose tissue) was extracted with petroleum ether (LabSynth, SP, Brazil) using a soxhlet apparatus during 96 hours and calculated by subtracting carcass weight before and after lipid extraction. The lean body mass was calculated as wet weight minus total lipid weight.

Determination of adipocyte area

Perigonadal white adipose tissue from male and female mice were dissected out and adipocytes were isolated according to the method of Rodbell (43), with minor modifications, as follows. Krebs Ringer bicarbonate buffer containing bovine serum albumin (3%), glucose (6 mM) and HEPES (25 mM), pH 7.4, was utilized. After collagenase treatment (1 mg/ml), isolated fat cells were filtered through a nylon mesh, washed two times, and the packed cells adjusted to approximately 10^5 cells/mL and placed on a Mallassez chamber for light microscopy and imaging capturing. Adipocyte sizes were analyzed with the software Image Pro Plus Analyser (Media Cybernetics, Silver Spring,

MD version 3.0). 200-500 cells from six mice per group for females and 400-700 cells from six mice per group for males were analyzed.

Mitochondrial respiratory rates

Mitochondria were isolated by conventional differential centrifugation (44) at 4°C. Liver homogenate was prepared in 250 mM sucrose, 1 mM EGTA, 10 mM Hepes buffer (pH 7.2) and 0.1% non esterified fatty acid free bovine serum albumin (BSA), and centrifuged at 600 g for 10 min. The supernatant was centrifuged at 7 000 g for 10 min. The pellet was washed in the same medium devoid of BSA and containing 0.1 mM EGTA. The final mitochondrial pellet was diluted in 250 mM sucrose to a protein concentration of 60 - 80 mg/ml, measured by the Biuret method and BSA as the protein standard. Oxygen consumption was measured using a temperature-controlled computer-interfaced Clark-type oxygen electrode from Hansatech Instruments equipped with magnetic stirring, at 28 °C. The experiments were done in standard medium containing 125 mM sucrose, 65 mM KCl, 4 mM potassium succinate, 2 mM inorganic phosphate, 1 mM magnesium cloryde, 0.4 mM EGTA and 10 mM Hepes buffer (pH 7.2). The state III respiration was initiated with addition of 200 nmol ADP/mg protein.

Insulin secretion from isolated pancreatic islets

The pancreas was removed and islets were isolated by handpicking after collagenase digestion. Groups of ten islets were first incubated for 30 min at 37°C in Krebs - bicarbonate buffer containing 2.8 mmol/l glucose and equilibrated with 95% O_2/ 5% CO_2 at pH 7.4. The solution was then replaced with fresh Krebs-bicarbonate buffer and islets were further incubated for 1h with medium containing 22.2 mmol/l glucose. The incubation medium contained (in mmol/l): 115.0 NaCl, 5.0 KCl, 24.0 $NaHCO_3$, 2.56 $CaCl_2$, 1.0 $MgCl_2$

and 0.3 % BSA (45). The insulin released after 1h was quantified as previously described (42) using rat insulin as the standard.

Statistical Analysis

The results are presented as the mean ± SEM for the number of determinations (n) indicated. The statistical analysis were done with the GraphPad InStat, Inc. software (version 3.00) using one way analysis of variance (ANOVA) followed by the Tukey post test for multiple comparisons or Student´s t test for two group comparisons. Statistical significance was defined as $P < 0.05$.

RESULTS

Male and female littermate mice overexpressing apo CIII combined or not with the natural promoter-driven CETP gene expression and control non-transgenic mice (CIII, CIII/CETP, CETP and NonTg) were placed on a high fat diet (14% fat by weight) during 20 weeks after weaning.

Table 1 shows plasma lipid levels in male mice from the 4 genotypic groups fed the high fat diet. They exhibited the expected lipemic phenotype, i.e., increased triglycerides (about 5 fold) and free fatty acids (30-50%) in apo CIII expressing groups (CIII and CIII/CETP) compared to the apo CIII non-expressing groups (CETP and NonTg). Interestingly, after 20 weeks on the high fat diet, TG and FFA levels are similar to those found in these mice on a chow diet, while cholesterol levels are significantly elevated in all groups (data not shown). Also as expected, plasma total cholesterol levels were reduced by 20-40% in CETP transgenic mice compared to the other groups. Similar results were found in female mice (data not shown).

Plasma insulin and glucose levels in fasting or fed states are shown in **Table 2**. Fasting glucose levels were neither affected by the high fat diet nor by the distinct

genotypes. However, male apo CIII transgenic mice showed significantly higher glycemia (35%) in the fed state, compared to the NonTg mice. This is likely ascribed to the lower insulin levels (45%) in these mice, which are also observed in apo CIII females. The lower insulin levels and insulin/glucose ratios in apo CIII expressing groups suggest impaired insulin secretion in the fed state. In fact, there was a significant reduction in the secretory capacity of the isolated pancreatic islet in response to high concentrations of glucose (22 mM): 2.1 ± 0.1 vs 2.8 ± 0.2 pg/islet/h, n=10-12, $p<0.02$, in CIII vs. NonTg islet, respectively.

Body mass and composition in male and female mice overexpressing apo CIII and/or CETP and NonTg mice are shown in **Table 3**. After 20 weeks on a high fat diet, the apo CIII transgenic mice had increased body weight in relation to both CETP expressing groups (CETP and CIII/CETP), a feature more marked in males. Lean body mass and water content of the mice carcasses were similar in the 4 groups of mice. Lipid content of the carcass, which mainly represents the subcutaneous adipose tissue, was higher in the apo CIII mice compared to the other groups. Particularly impressive is the effect of the CETP expression in the apo CIII background in reducing (25%) the subcutaneous adipose tissue mass to the levels of CETP and NonTg mice (**Fig. 1**).

Total visible visceral adipose tissue mass, including perigonadal, mesenteric and retroperitoneal depots were excised and weighed after the 20 week period of high fat diet, and are shown in **Figure 2**. Apo CIII mice had 1.5-2 fold greater visceral adipose tissue weight compared to the CETP and NonTg mice. The CIII/CETP group had an intermediate visceral adipose tissue weight, between CIII and CETP groups, showing that the CETP expression was not sufficient to correct the higher visceral adipose tissue weight of apo CIII transgenic mice. Thus, it is clear that the overexpression of apo CIII gene induced fat

accumulation in the visceral depots, in both sexes, as shown in pooled analysis (apo CIII expressing vs. non-expressing groups).

The adipocyte sizes from the four groups were measured in cells isolated from collagenase treated perigonadal adipose tissue and are shown in **Figure 3**. Adipocytes from apo CIII mice had significantly greater area (20-25%) than those from the other 3 groups. CETP expression in the apo CIII background completely normalized the larger adipocyte size of apo CIII mice.

Total lipid extracted from the livers showed no differences in hepatic fat content among the 4 groups (mg/g of liver): 68 ± 6, 57 ± 3, 66 ± 10, 62 ± 5, for CIII, CIII/CETP, CETP and NonTg male mice, respectively. Similar values were found in females.

Non-fasting plasma leptin levels (**Figure 4)** were lower in CETP transgenic groups (males and females) and higher in the apo CIII group (males), confirming the adipose tissue mass results. Leptin plasma levels paralleled better the pattern of subcutaneous adipose tissue mass observed in male and female mice, probably because the leptin production rates are higher for subcutaneous than for omental adipose tissue as verified in humans (46).

We previously reported that, under low fat chow diet, adult apo CIII transgenic mice exhibited an increased energy dissipating process measured as higher mitochondrial resting respiration, higher whole body metabolism and higher rectal temperature (37). This adaptation may explain why these mice, although hypertriglyceridemic, maintained their body weight and adipose tissue mass. Adipose tissue mass of apo CIII and NonTg male mice on a low fat diet are, respectively, 1.1 ± 0.3 and 0.9 ± 0.3% for perigonadal and 0.8 ± 0.1 and 0.8 ± 0.2 % for visible subcutaneous depots (% body weight). Since in the present experimental conditions of high fat diet, the apo CIII mice had higher body and adipose tissue mass, we re-evaluated their mitochondrial respiration rates (**Table 4**). It can be

observed that, under high fat diet, the apo CIII transgenic and NonTg mitochondria present similar resting respiration rates that are higher than the low fat diet fed NonTg mitochondria. Thus, high fat diet induced an increase in resting respiration in NonTg mitochondria but did not further increase the already higher mitochondrial resting respiration in apo CIII transgenic mice. Therefore, we can conclude that there is no differences in cell energy dissipation between high fat fed apo CIII and NonTg mice.

We also measured body core (rectal) temperature in the 4 genotypic groups as indicative of body metabolic rate (**Figure 5**). No statistical differences were observed among the groups, suggesting that the main differences observed in adipose tissue stores among these high fat fed groups are not related to differential body metabolic rates.

DISCUSSION

In this study we used transgenic mice overexpressing the human apo CIII gene, combined or not with the expression of the natural promoter driven CETP gene, to test the hypothesis that both proteins would prevent diet induced adipose tissue accumulation. Contrary to our hypothesis, the overexpression of apolipoprotein CIII increased diet induced visceral and subcutaneous adipose tissue stores. However, according to our hypothesis, the expression of CETP decreased the subcutaneous adipose tissue mass, visceral adipocyte size and plasma leptin levels of high fat fed apo CIII mice.

We considered the possibility that altered plasma concentrations of insulin, the most potent endogenous lipogenic hormone, could be responsible for the differential size of the lipid depots in these mice. Others and we have previously demonstrated that, although TG and FFA are elevated in apo CIII mice under low fat diet, these mice have normal glucose and insulin tolerance (47,48) and normal glucose stimulated insulin secretion by isolated

pancreatic islet (47). Here, we also report that under low fat diet they have adipose tissue stores similar to the controls. Since chronic consumption of high fat diet could lead to insulin resistance and compensatory hyperinsulinemia could increase adipogenesis (for review see 49), we measured glucose and insulin plasma levels in these high fat fed mice. Post-prandial glucose levels are higher while insulin levels are lower in apo CIII compared to NonTg mice. Reduced glucose stimulated insulin secretion in these apo CIII mice was confirmed in vitro in isolated pancreatic islet. Thus, plasma insulin levels are not implicated in the increased lipid stores in apo CIII mice; on the contrary, their adipose tissue sensitivity to insulin may be even increased.

Similar core body temperature in all groups and similar mitochondrial respiration rates in high fat diet fed CIII and NonTg mice suggest that there are no differences in body energy expenditure rates among these mice. Although we cannot exclude a differential spontaneous physical activity pattern, these data suggest that the lipid availability to adipose storage is most likely implicated in the distinct pattern of adiposity found in CIII and/or CETP expressing mice.

The previously reported protection from obesity conferred by the apo C1 overexpression was related to a 50% decrease in fatty acid uptake by white adipose tissue stores (39). On the other hand, lack of apo CIII significantly increased plasma TG lipolysis and fatty acid release to tissue uptake, leading to obesity (38). We show here that the overexpression of the apo CIII does not protect and actually exacerbated diet induced obesity. The increased plasma FFA concentrations observed in low fat fed apo CIII mice (12,47) suggested a saturated plasma FFA removal mechanism in these mice. However, lack of further increase in TG and FFA plasma levels after 20 weeks of high fat diet actually shows that these mice can handle the extra dietary fat supply suggesting no

saturation of plasma FFA tissue removal. Direct inhibitory effect of apo CIII on LPL activity is still elusive. While Ebara et al. (17) have shown an inhibitory effect of apo CIII mice plasma on the in vitro LPL activity, Aalto-Settala et al (12) have shown that VLDL from apo CIII and NonTg mice are equally lipolysed in vitro by purified LPL. The intravascular lower TG removal observed in apo CIII mice can be entirely explained by a decrease in the binding of apo CIII transgenic VLDL to a proteoglycan matrix model (21) or to glycosaminoglycan (17) where LPL is anchored. This delays, but does not necessarily inhibit VLDL-TG hydrolysis. The main reason these mice are hypertriglyceridemic is the decreased cellular uptake of the TG rich lipoprotein remnants (12,21). Hence, their prolonged residence time in plasma provides continuously more FFA to the peripheral tissues than if the remnant particles had been normally removed by liver. Thus, FFA availability to the adipose and other extra-hepatic tissues is actually increased in these apo CIII high fat fed mice. Additionally, Conde-Knape et al. (50) comparing the expression of apo CIII in apo B48-only or apo B100-only mice, found that apoB48 TG rich lipoproteins were more sensitive to apo CIII mediated inhibition of plasma clearance than apoB100 particles. Accordingly, the apoB48 particles were more resistant to TG enriching effect of apo CIII, since they remained longer in plasma and were more lipolysed. This reinforces the proposition that the apo CIII overexpression increases the FFA availability to the extra-hepatic tissues, especially from intestinal TG rich particles. Interestingly the apo B48/CIII mice were also hyperglycemic as we report here for the high fat fed apo CIII mice.

The CETP mediated lowering adipose tissue effect may be related to several processes. First, as a result of CETP mediated redistribution of TG into HDL particles, part of the FFA release is shifted to the liver since HDL-TG is a better substrate for hepatic lipase than for extra-hepatic LPL. In this way, less FFA would be available to the white

adipose tissue uptake in CETP expressing mice. This FFA flow shift from periphery to the liver may not be relevant when TG levels are low, as in CETP transgenic and NonTg mice; however, this may be very important when TG levels are markedly elevated, as in apo CIII expressing mice. Since there are no differences in liver fat content among all mice groups, the extra FFA taken up by the liver in CETP mice would have been metabolized or reutilized for VLDL secretion and recirculation. This could explain, at least in part, the adiposity reducing effects of the CETP expression in the apo CIII background.

Although the CETP transgenic line used in this work express very low levels of CETP in the adipose tissue (27), other possibilities that can be raised are local actions mediated by circulating CETP in decreasing TG accumulation and/or increasing lipolysis in adipose cells. Lay et al (51) have shown that enlarged adipocytes from several models of rodent obesity (Zucker rats, Fat mice and ob/ob mice) have reduced membrane cholesterol concentrations in different fat stores, demonstrating that lower cholesterol is characteristic of adipocyte hypertrophy per se. More importantly, reducing the cholesterol content of adipocytes via different ways (statins or cyclodextrins) modulates the expression of genes involved in energy metabolism such as upregulation of fatty acid synthase (FAS) and GLUT-1 and downregulation of GLUT-4 and UCP-3 in the adipocyte. These authors provided evidence that cholesterol might be a link between fat cell size and metabolic activity. Others and we have recently shown that CETP expression increases adipocyte uptake of exogenous cholesterol (30,32). Thus, by increasing cholesterol content of adipocytes, CETP could indirectly contribute to decrease FAS and increase UCP3 expression favoring a reduction in fat deposition in CETP expressing mice.

Other strong evidence for CETP mediated reduction in adipocyte size was recently provided by Zhou et al (36), using an adipose tissue specific promoter (aP2) driven CETP

transgenic mice. These mice presented physiological plasma concentrations of CETP, smaller adipocytes and reduced mRNA expression of the adipogenic genes LPL, PPARγ and SREBP-1c compared to controls. Thus, two independent CETP expressing mouse models show the same adiposity reducing effect of CETP.

Altogether the results presented here showed that while elevation of plasma apo CIII levels aggravates diet induced obesity, the expression of physiological levels of circulating CETP reverses this adipogenic effect, indicating a novel role for circulating CETP in modulating adiposity. Probable mechanisms seem to be related to the FFA availability to white adipose tissue uptake and to local expression of lipogenic genes.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Relative weight of subcutaneous adipose tissue depot (carcass lipid content, % BW) in female (A) and male (B) mice overexpressing apo-CIII and/or CETP and NonTg mice on a high fat diet for 20 weeks. Mean ± SEM (n=6). The same letters indicate the pair comparisons that are significantly different: a,b,c: $p<0.05$ (Student t test).

Figure 2. Relative weight of visceral adipose tissue depot (% BW) in female (A) and male (B) mice overexpressing apo-CIII and/or CETP and NonTg mice on a high fat diet for 20 weeks. Mean ± SEM (n=7-14). Black bars indicate pooled values from CIII expressing mice (+): CIII + CIII/CETP and CIII non-expressing mice (-): CETP + NonTg. The same letters indicate the pair comparisons that are significantly different: a,b: $p=0.052$ (ANOVA, females); d,e: $p<0.05$ (ANOVA, males); c,f: $p<0.01$ (Student t test).

Figure 3. Adipocyte size (μm^2) of perigonadal adipose depot in female (A) and male (B) mice overexpressing apo-CIII and/or CETP and NonTg mice after 20 weeks on a high fat diet. Mean ± SEM. 200-500 cells from six mice per group for females and 400-700 cells from six mice per group for males. The same letters indicate the pair comparisons that are significantly different: $p<0.001$ (ANOVA).

Figure 4. Non-fasting leptin plasma levels (ng/mL) in female (A) and male (B) mice overexpressing CIII and/or CETP and NonTg mice on a high fat diet for 20 weeks. Mean ± SEM (n=6). The same letters indicate the pair comparisons that are significantly different: $p<0.05$ (Student t test)

Figure 5. Rectal temperature (°C) in female (A) and male (B) mice overexpressing apo C-III and/or CETP and NonTg mice on a high fat diet for 20 weeks. Mean ± SE. n=7-14 for female and n=10-14 for male mice.

Table 1. Fasting plasma concentrations of lipids in male mice overexpressing apo CIII and/or CETP and NonTg mice after 20 weeks on a high fat diet.

	CIII	CIII/CETP	CETP	NonTg
CHOL (mg/dL)	265 ± 20^{a} (10)	220 ± 20^{b} (12)	$144 \pm 10^{a,b}$ (11)	200 ± 15 (12)
TG (mg/dL)	$530 \pm 40^{c,d}$ (10)	$525 \pm 26^{e,f}$ (12)	$95 \pm 10^{d,f}$ (11)	$72 \pm 5^{c,e}$ (12)
FFA (mmol/L)	1.8 ± 0.3^{g} (10)	1.8 ± 0.2^{h} (12)	1.4 ± 0.1 (11)	$1.1 \pm 0.1^{g,h}$ (12)

CHOL: Cholesterol, TG: Triglycerides, FFA: free fatty acid. Values represent means ± SEM (n). The same letters indicate the pair comparisons that are significantly different: *a,c,d,e,f:* $p<0.001$; *b,g,h:* $p<0.05$, ANOVA.

Table 2: Plasma glucose (mg/dL) and insulin (ng/mL) levels in fasted and fed male and female mice overexpressing apo CIII and/or CETP and NonTg mice after 20 weeks on a high fat diet.

		CIII	CIII/CETP	CETP	NonTg
Glucose (mg/dL) (Fasted)	Male	98 ± 10 (10)	73 ± 6.0 (12)	75 ± 7.0 (11)	87 ± 8.0(12)
	Female	74 ± 7.0 (6)	73 ± 6.0 (11)	65 ± 7.0 (8)	65 ± 4.0(12)
Glucose (mg/dL) (Fed)	Male	371 ± 26 (10)[a]	299 ± 25 (12)	297 ± 19 (12)	277 ± 14(13)[a]
	Female	245 ± 22 (7)	243 ± 20 (11)	225 ± 8 (10)	230 ± 9(14)
Insulin (pg/mL) (Fed)	Male	693 ± 78 (7)[b]	731 ± 102 (8)[c]	942 ± 213 (7)	1299 ± 173(7)[b,c]
	Female	641 ± 50 (6)[d,e,f]	1093 ± 158 (7)[d]	1160 ± 189 (6)[e]	1035 ± 146(6)[f]
Insulin/glucose (Fed)	Male	1.93 ± 0.3 (7)[g]	2.70 ± 0.4 (8)[h]	3.15 ± 0.6 (7)	4.90 ± 0.5(7)[g,h]
	Female	2.81 ± 0.3 (6)[i,j]	4.96 ± 1.1 (7)	5.28 ± 1.0 (6)[i]	4.84 ± 0.7(6)[j]

Values represent means ± SEM (n). The same letters indicate the pair comparisons that are significantly different: $p<0.05$: *a, b, g, h* (ANOVA) and *c, d, e, f, i, j* (Student t test).

Table 3: Body mass and composition (% body weight) in male and female mice overexpressing apo CIII and/or CETP and NonTg mice after 20 weeks on a high fat diet.

	Genotype	Body weight (g)	Lean body mass	Water	Lipid *
MALE					
	CIII	38.5 ± 1.8[a,b]	50.0 ± 1.0	35.0 ± 1.0	21.5 ± 1.1[c,d]
	CIII/CETP	31.0 ± 1.6[a]	55.0 ± 2.4	38.0 ± 1.6	15.2 ± 1.7[c]
	CETP	31.5 ± 1.7[b]	56.1 ± 3.0	39.0 ± 2.2	15.0 ± 2.3[d]
	NonTg	33.0 ± 2.5	54.0 ± 2.8	38.5 ± 2.0	16.0 ± 2.5
FEMALE					
	CIII	27.0 ± 1.5	56.0 ± 2.0	39.0 ± 1.3	16.0 ± 1.7[e]
	CIII/CETP	24.0 ± 0.8	60.0 ± 0.7	41.5 ± 0.5	11.2 ± 0.8[e]
	CETP	24.3 ± 0.5	60.0 ± 1.0	41.0 ± 0.9	11.2 ± 1.1
	NonTg	25.0 ± 2.2	57.0 ± 2.7	39.5 ± 1.7	13.5 ± 2.4

∗ lipid extracted from mice carcasses indicate subcutaneous lipid depots. Values represent means ± SEM for six animals in each group. The same letters indicate the pair comparisons that are significantly different: $p<0.05$ (Student t test)

Table 4. Effect of high fat diet on the mitochondrial respiration rates in liver mitochondria isolated from apo CIII transgenic and NonTg mice.

	Phosphorylating respiration (State III)	Resting respiration (State IV)	Respiratory control (state III/IV)
CIII (high fat diet)	140.4 ± 4.7	34.8 ± 0.7[a]	4.0 ± 0.08[d]
NonTg (high fat diet)	138.5 ± 2.6	33.9 ± 1.2[b]	4.1 ± 0.12[c]
NonTg (low fat diet)	128.9 ± 4.3	27.5 ± 0.3[a,b]	4.6 ± 0.10[c,d]

Mean ± SEM (n = 9). Respiration rates given in nanoatoms oxygen/mg protein/min. The state III respiration was initiated with addition of 200 nmol ADP/mg protein. The same letters indicate the pair comparisons that are significantly different: *a,b,c,d:* $p<0.05$ (ANOVA).

Figure 1.

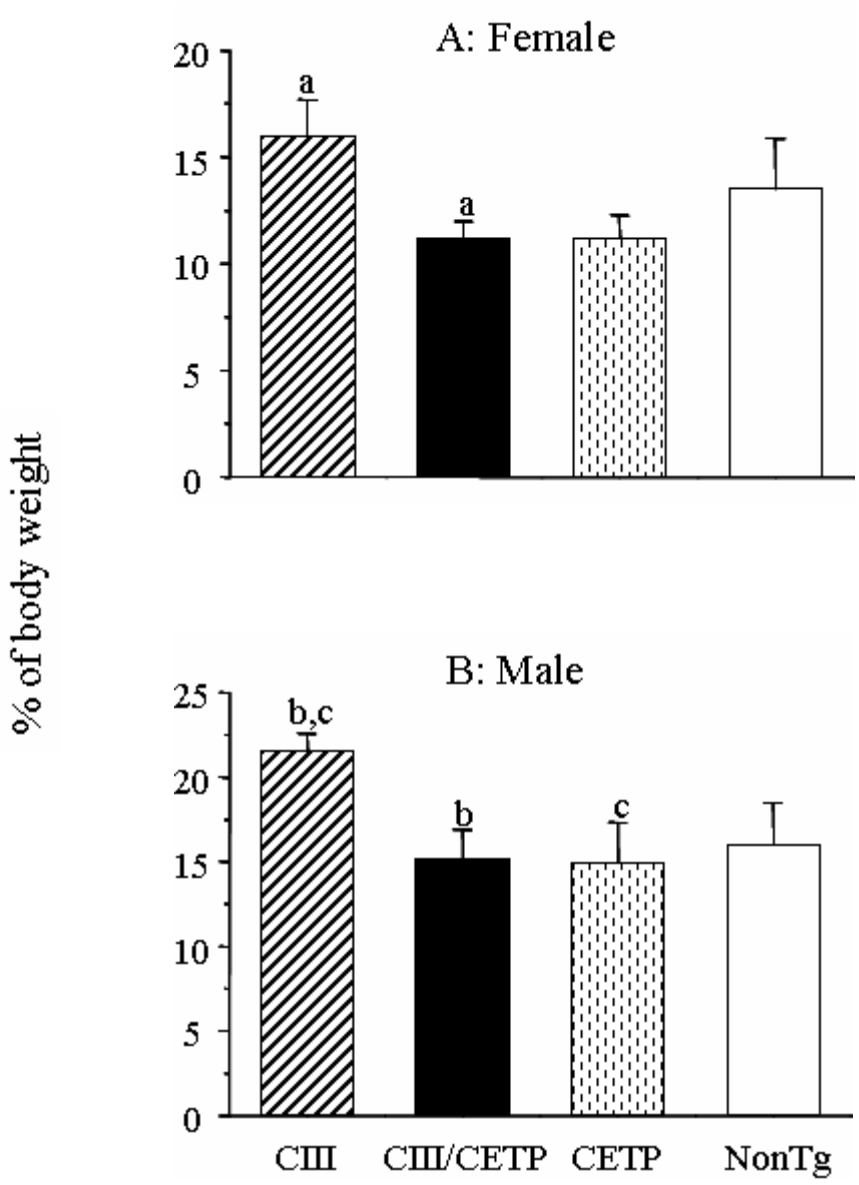

A: Female
B: Male
% of body weight
20
15
10
5
0
25
a
a
b,c
b
c
CIII
CIII/CETP
CETP
NonTg

Figure 2.

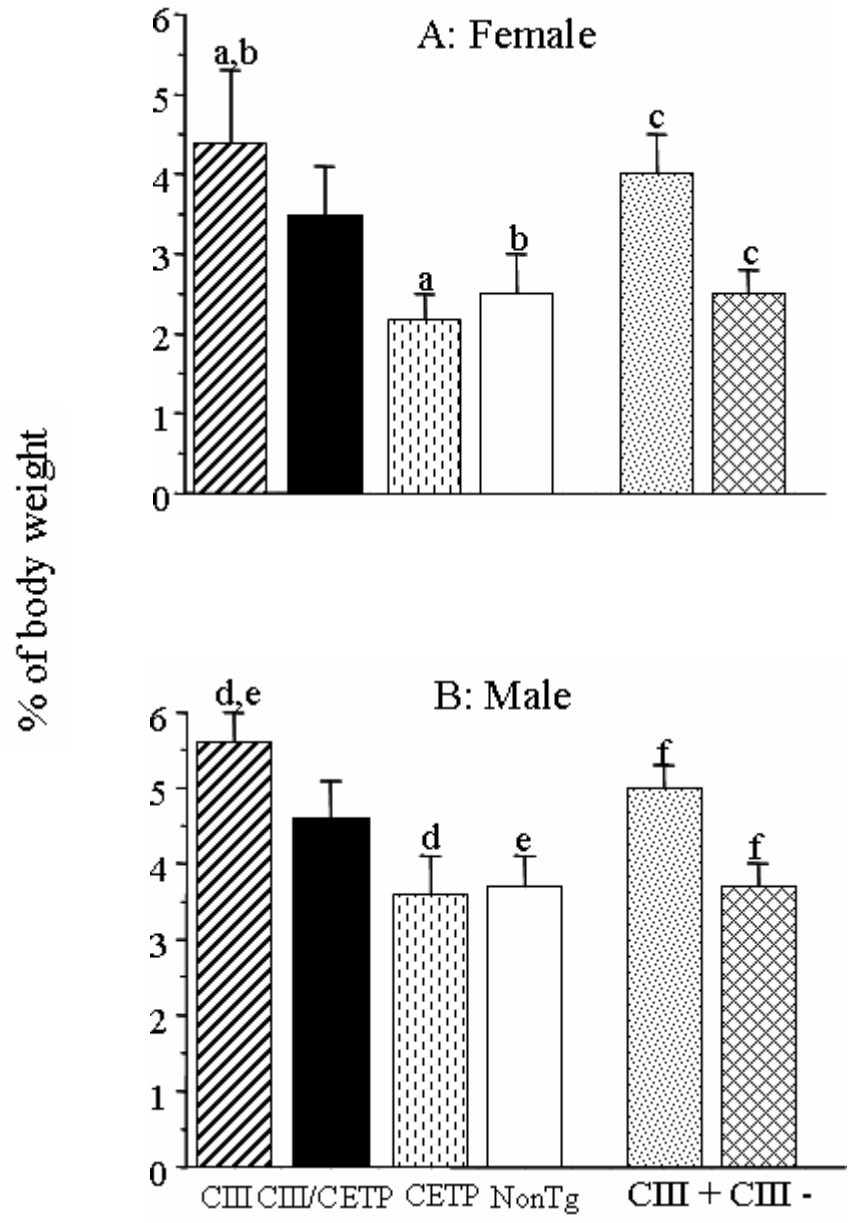

A: Female
B: Male
% of body weight
a,b
a
b
c
c
d,e
d
e
f
f
0
1
2
3
4
5
6
CIII
CIII/CETP
CETP
NonTg
CIII + CIII -

Figure 3.

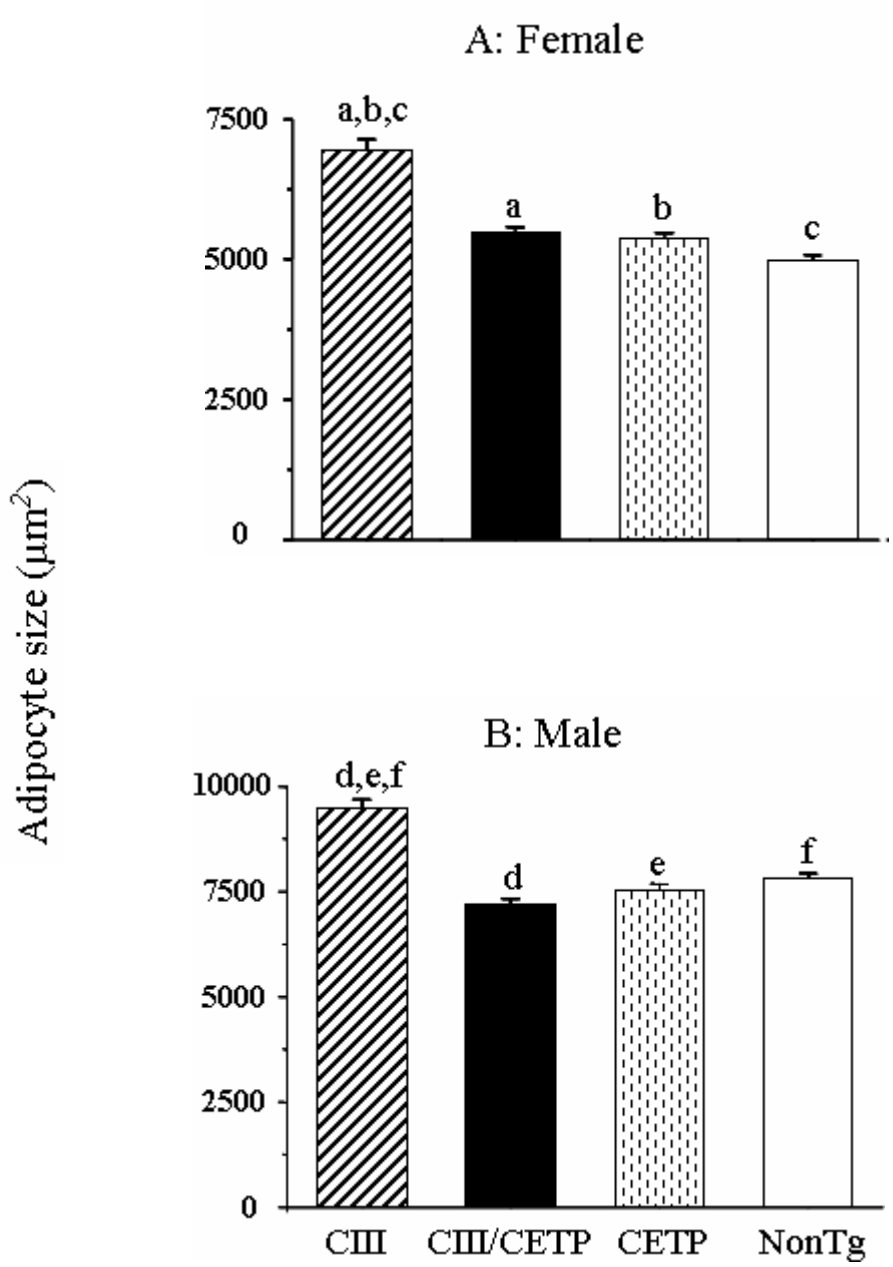

A: Female
7500
5000
2500
0
a,b,c
a
b
c
B: Male
10000
7500
5000
2500
0
d,e,f
d
e
f
Adipocyte size (μm²)
CIII
CIII/CETP
CETP
NonTg

Figure 4.

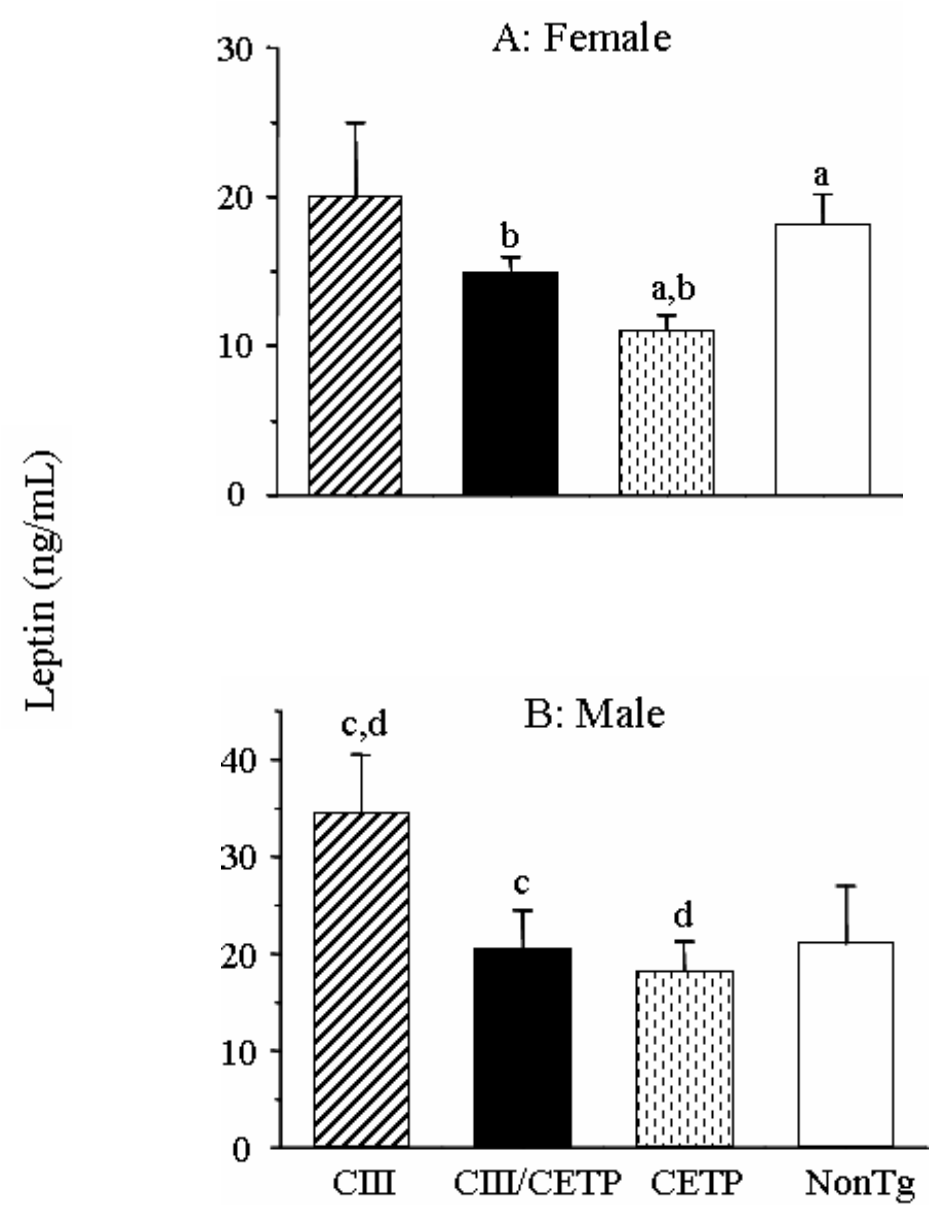
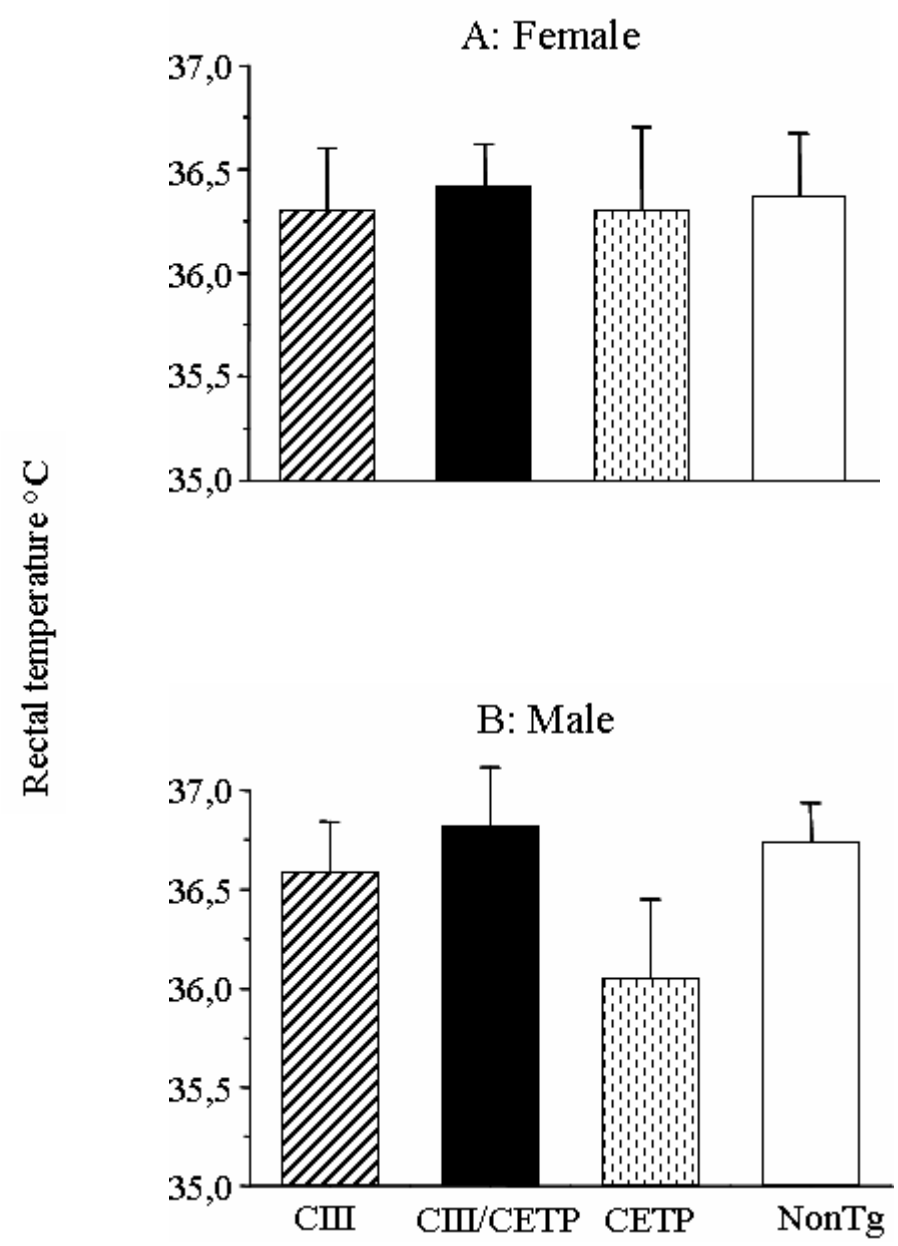

A: Female
B: Male
Leptin (ng/mL)
30
20
10
0
40
30
20
10
0
b
a,b
a
c,d
c
d
CIII
CIII/CETP
CETP
NonTg

Figure 5.


A: Female
37,0
36,5
36,0
35,5
35,0
Rectal temperature °C
B: Male
37,0
36,5
36,0
35,5
35,0
CIII
CIII/CETP
CETP
NonTg

VI - CONCLUSÕES E PERSPECTIVAS:

Na primeira parte deste trabalho, verificamos que a expressão da CETP modifica o metabolismo intravascular de triglicérides de origem intestinal da seguinte maneira:

1) aumenta a trigliceridemia pós-prandial;

2) não altera a secreção hepática de VLDL-TG e VLDL-COL, tanto no jejum como 5 horas após sobrecarga oral de gorduras;

4) não altera a absorção intestinal de gorduras;

3) retarda a remoção plasmática de TG e COL de lipoproteínas artificiais ricas em TG;

5) reduz significativamente a atividade da lipoproteína lipase periférica.

Estes dados sugerem que, dependendo da quantidade de CETP no plasma, o organismo pode ser mais intolerante à gordura da dieta, acumular maior quantidade de remanescentes de lipoproteínas ricas em TG no compartimento plasmático, as quais são partículas aterogênicas.

Na segunda parte deste trabalho, verificamos que as expressões da apolipoproteína CIII e da CETP alteraram a formação de depósitos adiposos regionais em camundongos geneticamente modificados alimentados com dieta rica em gordura por longo prazo. Podemos resumir nossos achados da seguinte maneira:

<u>Efeitos da super-expressão da CETP em transgênicos para apoCIII</u>

1- redução de gordura perigonadal e subcutânea;

2- redução do tamanho do adipócito;

3- redução da concentração plasmática de leptina.

<u>Efeitos da super-expressão de apo CIII</u>

1- não afeta o tamanho dos depósitos adiposos viscerais e subcutâneos na vigência de dieta pobre em gordura;

2- causa aumento dos depósitos adiposos viscerais e subcutâneos e do tamanho dos adipócitos viscerais e concentração de leptina em adultos, mediante dieta rica em gordura.

Esses achados mostraram que o aumento do apo CIII agrava a obesidade induzida por dieta rica em gordura e que concentrações fisiológicas de CETP revertem o efeito adipogênico da apo CIII, indicando uma nova função desta proteína na modulação da adiposidade.

Como perspectivas de continuidade deste trabalho, podemos apontar duas aplicações:

1- Estudar os polimorfismos do gene da CETP associados com maior ou menor atividade desta proteína na população humana, testar se estes polimorfismos estão também associados à tolerância à gordura da dieta. Em caso positivo, usar a identificação destes polimorfismos para tratamento ou aconselhamento dietético.
2- Desenvolver uma ferramenta terapêutica para aumentar a atividade da CETP para auxiliar o tratamento de indivíduos obesos.

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Cid. Univ. "Zeferino Vaz", 05 de novembro de 2001

Ofic. CIBio 07/2001

Ilma. Sra.
Profa. Dra. Helena Franco C. Oliveira
Departamento de Fisiologia e Biofísica
Instituto de Biologia - UNICAMP

Prezada Professora,

Este é para lhe informar que os Projetos abaixo relacionados, do qual V.Sa. é o Pesquisador responsável, foi aprovado pela CIBio em sua décima segunda reunião ordinária, ocorrida em 26 de outubro pp, para ser desenvolvido nas dependências do Departamento de Fisiologia e Biofísica. Solicito especial atenção ao prazo para o envio de relatório, que deve seguir o modelo constante do Manual da CIBio (vide http://www.unicamp.br/ib/CIBio/index.html).

No. Projeto	Data de recepção	Nome do Projeto	Prazo para envio de relatório à CIBio
CIBio2001/06	28/08/2001	Bioenergética mitocondrial em camundongos transgênicos hiperlipidêmicos	07/09/2002
CIBio2001/07	28/08/2001	Metabolismo de lipoproteínas ricas em triglicérides em camundongos que expressam CETP	07/09/2002

Sem mais para o momento, subscrevo-me,

Atenciosamente,

Wirla M S C Tamashiro
Profa. Dra. Wirla M S C Tamashiro
Presidente da CIBio/IB/UNICAMP

C/C ao Chefe do Departamento

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Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **485-1**, sobre **"Efeito da Expressão da CETP sobre o Metabolismo das Lipoproteínas Ricas em Triglicerídeos e Adiposidade em Camundongos Transgênicos"** sob a responsabilidade de **Helena Coutinho Franco de Oliveira** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 28/11/2002.

CERTIFICATE

We certify that the protocol nº **485-1**, entitled **"Effect of CETP Expression on the Triglyceride Rich Lipoprotein Metabolism and Adiposity in Transgenic Mice"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on November 28, 2002.

Campinas, 28 de Novembro de 2002.

Profa. Dra. Liana Verinaud
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

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