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ABSTRACT

The acute-phase protein haptoglobin (Hpt) binds apolipoprotein A-I (ApoA-I) and impairs its action on lecithin-cholesterol acyltransferase, an enzyme that plays a key role in reverse cholesterol transport. We have previously shown that an ApoA-I mimetic peptide, P2a, displaces Hpt from ApoA-I, restoring the enzyme activity in vitro. The aim of this study was to evaluate whether P2a displaces Hpt from ApoA-I in vivo and whether this event leads to anti-inflammatory activity. Mice received subplantar injections of carrageenan. Paw volume was measured before the injection and 2, 4, 6, 24, 48, 72, and 96 h thereafter. At the same time points, concentrations of HDL cholesterol (C) and cholesterol esters (CEs) were measured by high-performance liquid chromatography, and Hpt and ApoA-I plasma levels were evaluated by enzyme-linked immunosorbent assay. Western blotting analysis for nitric-oxide synthase and cyclooxygenase (COX) isoforms was also performed on paw homogenates. CEs significantly decreased in carrageenan-treated mice during edema development and negatively correlated with the Hpt/ApoA-I ratio. P2a administration significantly restored the CE/C ratio. In addition, P2a displayed an anti-inflammatory effect on the late phase of edema with a significant reduction in COX2 expression coupled to an inhibition of prostaglandin E2 synthesis, implying that, in the presence of P2a, CE/C ratio rescue and edema inhibition were strictly related. In conclusion, the P2a effect is due to its binding to Hpt with consequent displacement of ApoA-I that exerts anti-inflammatory activity. Therefore, it is feasible to design drugs that, by enhancing the physiological endogenous protective role of ApoA-I, may be useful in inflammation-based diseases.

Introduction

Homeostasis of cholesterol (C) is essential for cell function and survival because cholesterol is toxic when it accumulates in the plasma membrane or within the cells. In atherogenesis, a critical role is played by the process known as reverse cholesterol transport (RCT), through which the accumulated cholesterol is transported from the vessel wall to the liver for excretion. This process includes acceptors such as high-density lipoproteins (HDLs), apolipoprotein A-I (ApoA-I), apolipoprotein E, lecithin-cholesterol acyltransferase (LCAT) (EC 2.3.1.43), and several other enzymes (Rader, 2006; Rader et al., 2009). Evidence has suggested a protective role for HDLs in atherosclerosis based on their ability to promote RCT (Rader, 2006). At present, it is still unclear whether part of the “protective” effect of HDLs is due to functions beyond RCT and whether these functions could be enhanced or reduced after the interaction with circulating proteins. Indeed, it is known that HDLs undergo dramatic modification in structure and composition as a result of the concerted actions of the acute-phase response protein and inflammation (Khovidhunkit et al., 2004; Esteve et al., 2005). In these conditions, HDL particles progressively lose normal biologi-
The critical role of inflammation at all stages of atherosclerosis is now recognized, including triggers, mediators, and end-effectors (Kontush and Chapman, 2006; Libby, 2007). In several studies, we have shown that the acute-phase protein haptoglobin (Hpt) binds the HDL protein components ApoA-I and apolipoprotein E, impairing their key functions in RCT (Porta et al., 1999; Spagnoolu et al., 2005; Salvatore et al., 2007, 2009; Cigiano et al., 2009). Haptoglobin is a polymorphic glycoprotein that exhibits phenotype prevalence in cardiovascular diseases (Delanghe et al., 1997). Its circulating levels are enhanced during the acute phase of inflammation to capture and transport free hemoglobin to the liver (Langois and Delanghe, 1996). ApoA-I, the major protein component of HDL, plays a key role in reverse cholesterol transport. This protein stimulates the efflux of cellular cholesterol and activates the enzyme LCAT, which in turn converts the free cholesterol into cholesteryl esters (CEs), addressing them to HDLs for transport into the circulation (Rader and Daugherty, 2008). The binding of Hpt to ApoA-I is associated with inhibition of LCAT activity and reduction of ApoA-I-mediated delivery of cholesterol to hepatocytes leading to 1) poor cholesterol removal from peripheral cells and 2) a low level of HDL cholesterol in the circulation (Spagnoolu et al., 2005; Salvatore et al., 2007; Cigiano et al., 2009). Moreover, several studies have confirmed that high levels of Hpt are associated with increased risk of developing cardiovascular events or myocardial infarction (Braeckman et al., 1999; De Bacquer et al., 2001; Matuszek et al., 2003). We have previously shown that an ApoA-I mimetic peptide with an amino acid sequence overlapping the stimulatory site for LCAT (P2a: acetyl-LSPLGEEGRDRARRHVDALRTHL-amide) efficiently displaced Hpt from ApoA-I. In addition, in an in vitro setting, P2a was able to rescue the stimulatory function of ApoA-I in the presence of high Hpt levels, whereas, when incubated without Hpt, it did not affect LCAT cholesterol esterification (Spagnoolu et al., 2005). Because the anti-inflammatory activity of HDLs and ApoA-I has been well documented (Kontush and Chapman, 2006; Rader, 2006; Gomaraschi et al., 2008; Sherman et al., 2010), we hypothesized that the ApoA-I mimetic peptide P2a could also be effective in vivo in displacing Hpt from ApoA-I, leaving this apolipoprotein available for anti-inflammatory activity. In this study, we show that the peptide P2a rescues LCAT-dependent cholesterol esterification in vivo causing, in addition, an anti-inflammatory effect.

**Materials and Methods**

**Drugs and Reagents.** Sheep anti-ApoA-I and sheep anti-Hpt polyclonal antibody were purchased from Serotec (Oxford, UK). [1a,2a-3H](Cholesterol (45 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). SIL G plates for thin-layer chromatography (thickness 0.25 mm) were obtained from Macherey-Nagel (Düren, Germany). Chemicals of the highest purity, bovine serum albumin (BSA), cholesterol, cholesterol esterified linoleate, donkey anti-sheep IgG horseradish-linked, o-phenylenediamine, dextran sulfate (Dextralip 50), carrageenan, molecular mass markers, and SUPELCOSIL LC-18 (5-μm particle size, 250 x 4.6 mm i.d.) were obtained from Sigma-Aldrich (St. Louis, MO). Polystyrene 96-well plates were purchased from Nunc (Roskilde, Denmark). Bradford reagent was obtained from Bio-Rad Laboratories (Milan, Italy). The antibodies against COX2 and iNOS were from Transduction Laboratories (Lexington, KY). [3H]PGE2 was from Perkin-Elmer (Milan, Italy). All other reagents and compounds used were obtained from Sigma-Aldrich (Milan, Italy).

**Peptide Synthesis.** The peptide P2a (ApoA-I sequence from L141 to A164; acetyl-LSPLGEEGRDRARRHVDALRTHL-amide) and the scramble peptide control (P2as: acetyl-RLSARLTLHGEPVALDEMRADRHA-amide) were solid phase-synthesized using PAL-PEG-P5 resin (0.16 mmol/g) (Applied Biosystems, Foster City, CA) by standard N-(9-fluorenyl) methoxycarbonyl chemistry. Amino acid coupling was performed using a 10 M excess of N-(9-fluorenyl) methoxycarbonyl-amino acid, 9. Eq of 2-(1H-benzo triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-1-hydroxybenzo triazole, and 20 Eq of N,N-diisopropylethylamine. A solution of 30% piperidine in N,N-dimethylformamide was used in the deprotection step (two 5-min washes). The coupling reaction was performed for 60 min followed by a 10-min acylation step with acetic anhydride (2 M-N,N-diisopropylethylamine (0.55 M)-1-hydroxybenzo triazole (0.06 M) in N-methylpyrrolidone. Cleavage from the resin was achieved by treatment with trifuoroacetic acid, triisopropyl silane, ethanol, and water (94:1:2.5:2.5, v/v/v/v) at room temperature for 3 h. Peptide analysis and purification were performed by reverse-phase-HPLC on a C18 Proteo column (Phenomenex, Torrance, CA) using a gradient of CH3CN (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) going from 20 to 50% in 30 min. Peptide identities were assessed by electrospray ionization mass spectrometry on a Thermo Finnigan MSQ liquid chromatograph-mass spectrometer (P2a: molecular mass experimental 2755.5 Da, molecular mass calculated 2756.1 Da; P2as: molecular mass experimental 2755.5 Da, molecular mass calculated 2756.1 Da).

**Mouse Paw Edema.** Male Swiss mice (CD-1; Harlan, Correzzana, Italy) weighing 28 to 30 g were used for in vivo experiments. The experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, publication 86-23, revised 1985) as well as the specific guidelines of the Italian (N.116/1992) and European Council law (N.86/609/CEE). Animals were divided into groups (n = 6/group) and lightly anesthetized with isoflurane. Each group of animals received subplantar injections of 50 μl of carrageenan 1% (v/v) or 50 μl of vehicle (saline) in the left hind paw. Paw volume was measured by using a hydroplethysmometer specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before the subplantar injection and 2, 4, 6, 24, 48, 72, and 96 h thereafter. The same operator always performed the double-blind assessment of paw volume. The increase in paw volume was calculated as the difference between the paw volume measured at each time point and the basal paw edema. Each group of animals received intraperitoneal (100 μl) administration of P2a peptide (0.3, 0.6, or 1 mg/kg), P2as peptide (1 mg/kg), or vehicle (saline). All peptides were administered immediately before the injection of carrageenan and 24 h thereafter.

**ApoA-I and Hpt Immunoreasay.** The concentration of ApoA-I and Hpt was determined by ELISA using mouse antigens as standards for calibration, isolated as reported previously (Spagnoolu et al., 2003) and exhibiting more than 98% purity by electrophoresis and densitometry analysis. Aliquots of plasma (50 μl from 1:1000, 1:10,000, 1:20,000, 1:45,000, 1:60,000, and 1:100,000 dilutions) were diluted in coating buffer (7.3 mM Na2CO3, 17 mM NaHCO3, and 1.5 mM NaN3, pH 9.6), loaded into the wells of a microtiter plate, and processed essentially according to a published procedure (Cigiano et al., 2005). In particular, sheep IgG (anti-ApoA-I or anti-Hpt, respectively) was used as the primary antibody and donkey anti-sheep IgG horseradish-linked IgG as the secondary antibody for color development. Measurements were performed using a calibration curve, obtained by determining the immunoreactivity of 1, 0.5, 0.25, 0.125,
with 30% ZnSO₄ for 15 min (Thomsen et al., 1990). PGE₂ was determined in deproteinized exudates by radioimmunoassay as mean

To determine PGE₂ levels, proteins were removed from the exudates using 50 mM MnCl₂ and 0.1% dextran sulfate (50 kDa) according to a published procedure to remove very-low-density lipoprotein and low-density lipoprotein (Burstein et al., 1970). After this treatment, two 25-

The residue was dissolved in 0.2 ml of acetonitrile-isopropanol (57:43, v/v), and 20 μl were processed by reverse-phase HPLC. Chromatography was performed on a C18 column at 40°C with a 1 ml/min flow rate, according to a published procedure (Cigliano et al., 2005). The amounts of unesterified and total cholesterol (HDL-C) were measured in samples processed without and with KOH, respectively, and the amount of CE was measured in samples processed with KOH. After incubation, both mixtures were supplemented with 0.15 μl of 1% NaCl and, after addition of 2 ml of ice-cold hexane, were vigorously shaken for 2 min. The hexane extract was added, and the lower phase was likewise extracted two more times. The three extracts were pooled and dried under a nitrogen stream. The CE/C ratio in HDLs was determined by injecting different amounts (n = 12) of standard C, were used for quantitative analysis.

The HDL fraction was isolated from plasma of carrageenan-treated mice (21.5 ± 1 at time 0 versus 12 ± 0.6 at 24 h, p < 0.01; 21.5 ± 1 at time 0 versus 10.5 ± 0.9 at 48 h, p < 0.01) (Fig. 1), whereas no change was detected in controls. LCAT activity in vitro was not significantly modified by carrageenan injection at the time points tested (data not shown). The levels of apolipoprotein A-I, the protein that stimulates the enzyme LCAT, were found to be unchanged in inflamed mice (Fig. 2). These results suggest that, in carrageenan-treated mice, a plasma factor influences the cholesterol esterification, and this effect is not assessable in vitro.

**Correlation between the Ratio of Hpt/ApoA-I and CE/C.** Haptoglobin binds ApoA-I, the main stimulator of LCAT, thus inhibiting the enzyme activity and the efficiency of the reverse cholesterol transport (Spagnuolo et al., 2005; Salvatore et al., 2007; Cigliano et al., 2009). The plasma level of Hpt was measured in carrageenan-treated mice (Fig. 2). The change in Hpt level after carrageenan injection showed a biphasic trend: an early increase peaking 2 h after the carrageenan injection (29.1 ± 1.5 μM in inflamed mice versus 14.1 ± 0.5 μM in controls; p < 0.01), returning to the physiological values at 144 h (14.2 ± 3 μM in inflamed mice versus 13.4 ± 2.1 μM in controls). The Hpt/ApoA-I ratio variance followed by the Bonferroni test for multiple comparisons or t test analysis where appropriate, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when p < 0.05. Each sample was processed at least in triplicate.

**Results**

**Cholesterol Esterification Is Reduced in Mouse Edema.** The HDL fraction was isolated from plasma of carrageenan-treated mice after 2, 4, 6, 24, 48, 72, 96, and 144 h. The molar concentration of C and CEs was measured, and the molar ratio CE/C was calculated as an index of LCAT activity ex vivo (Subbaiah et al., 1997; Purbee et al., 2001).

In the early phase of carrageenan-induced inflammation (0–6 h), no differences were found in the CE/C ratio compared with that for the vehicle (data not shown). In the late phase of inflammation (24–48 h), the values of the CE/C ratio were found to be significantly decreased in carrageenan-treated mice (21.5 ± 1 at time 0 versus 12 ± 0.6 at 24 h, p < 0.01; 21.5 ± 1 at time 0 versus 10.5 ± 0.9 at 48 h, p < 0.01) (Fig. 1), whereas no change was detected in controls. LCAT activity in vitro was not significantly modified by carrageenan injection at the time points tested (data not shown). The levels of apolipoprotein A-I, the protein that stimulates the enzyme LCAT, were found to be unchanged in inflamed mice (Fig. 2). These results suggest that, in carrageenan-treated mice, a plasma factor influences the cholesterol esterification, and this effect is not assessable in vitro.

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was found to be negatively correlated with the CE/C ratio \( (p < 0.01; r = 0.94) \) (Fig. 3). These data fit well with an inhibitory role of Hpt in the ApoA-I-dependent activity of cholesterol esterification on HDLs.

**P2a Peptide Inhibits Mouse Paw Edema in a Dose-Dependent Manner.** In the early phase of carrageenan-induced paw edema (0–6 h), no differences were found among groups treated with P2a at all doses tested compared with the vehicle group (Fig. 4). In the late phase of edema (24–96 h), injection of P2a caused a significant and dose-dependent inhibition of edema (Fig. 4). The specificity of the P2a effect was confirmed by the finding that the P2as did not affect edema development in both phases (Fig. 4).

**P2a Peptide Restores Cholesterol Esterification.** It is known that the binding of Hpt to ApoA-I is associated with reduced LCAT activity. We previously found that, in vitro, the peptide P2a, homologous to the ApoA-I sequence from Leu141 to Ala164, displaces Hpt from ApoA-I, restoring the activity of LCAT (Spagnuolo et al., 2005). To evaluate whether the P2a peptide displaces Hpt in vivo, different doses of P2a (0.3, 0.6, or 1 mg/kg i.p.) or of P2as were injected in mice. When mice were treated with 0.6 or 1 mg/kg P2a, the CE/C ratio was found to be significantly restored (Fig. 5A). No effect of P2a on HDL-C was found (Fig. 5B). This result shows that P2a, administered intraperitoneally, efficiently engages Hpt, thus restoring ApoA-I function also in vivo.

**P2a Peptide Reduces COX2 Expression and PGE2 Generation.** P2a exerted its anti-inflammatory action in the second phase of carrageenan-induced paw edema. To identify the molecular mechanisms responsible for this anti-inflammatory effect, the expressions of inducible isoforms of nitric-oxide synthase and COX, i.e., iNOS and COX2, were evaluated at 24 and 48 h after carrageenan administration. As shown in Fig. 6, Western blot analysis did not reveal any significant alteration in COX2 expression at 24 h from edema induction in all three groups of mice treated with P2a. In contrast, 48 h after carrageenan injection, P2a (1 mg/kg) significantly reduced COX2 expression (Fig. 6). The COX2 involvement was also confirmed by the significant reduction of PGE2 levels in paw exudates obtained from mice treated with P2a (Fig. 7). P2a administration did not affect iNOS and eNOS expression at any time point (supplemental figure).

**Discussion**

It is known that the binding of Hpt to ApoA-I is associated with reduced LCAT activity in vitro (Spagnuolo et al., 2005). This binding, by decreasing the amount of free ApoA-I available for LCAT stimulation, impairs cholesterol esterification (Spagnuolo et al., 2005; Salvatore et al., 2007, 2009; Cigliano et al., 2009). The peptide P2a, which presents the ApoA-I amino acidic sequence overlapping the domain required for LCAT stimulation, displaces Hpt from ApoA-I and rescues the enzyme activity in vitro (Spagnuolo et al., 2005). However, it has still not been determined whether this mechanism is relevant in vivo. To address this issue, we tested whether P2a could exert similar activity in vivo. By monitoring the plasma CE/C ratio and Hpt levels during edema development, we found an inverse correlation between CE/C
The inflammatory response induced by carrageenan in mice is coupled with a significant increase in Hpt levels (Salvatore et al., 2007). Therefore, in this in vivo experimental model, Hpt acts as a competitive inhibitor of ApoA-I for LCAT activity, causing a reduction in cholesteryl ester production and confirming the physiopathological relevance of this mechanism in inflammation. In the late phase of mouse paw edema, P2a displayed a clear anti-inflammatory effect. This effect did not involve changes in ApoA-I levels or LCAT activity, as confirmed by the finding that these two latter parameters were not modified in plasma harvested by mice treated with carrageenan. Thus, we hypothesize that Hpt, by binding ApoA-I, impairs LCAT function. In other words, as summarized for clarity in Fig. 8, the effect of P2a relies on the boosting of an endogenous mechanism, e.g., Hpt capture, rather than on a direct anti-inflammatory action. This hypothesis is sustained by the finding that systemic administration of P2a dose dependently increased the CE/C ratio, rescuing it to almost physiological levels at the higher dose tested (1 mg/kg). The specificity of the P2a effect was confirmed by the experiments performed using a scrambled peptide, for which the mismatching of amino acid positions led to a lack of efficacy. This effect of P2a on LCAT activity was paralleled by an anti-inflammatory effect on the late phase of carrageenan-induced paw edema (24–96 h). It is well known that in this mouse model of edema COX2-derived eicosanoids and/or iNOS- or eNOS-derived nitric oxide is involved at different time points (Posadas et al., 2004). P2a administration did not modulate eNOS expression. These findings correlate well with the lack of activity of P2a in the early phase of the edema. In the late phase, in which P2a significantly modifies the cholesterol biochemical pathway, there was also a reduction in COX2 expression as well as of PGE2 levels. This finding implies...
that the two phenomena observed in presence of P2a, i.e., CE/C ratio rescue and edema inhibition, are related.

Our data clearly imply that P2a, by virtue of its ability to bind Hpt, makes available more ApoA-I that acts as an endogenous anti-inflammatory signal. Indeed, the P2a anti-inflammatory effect is evident at the same time point at which recovery of the CE/C ratio occurs, i.e., 48 h after carrageenan injection. This hypothesis is further supported by the fact that P2a significantly inhibits exclusively the second phase of the edema at which the Hpt level reaches its maximum.

In conclusion, we have demonstrated that P2a, an ApoA-I-derived peptide with an amino acid sequence overlapping the stimulatory site for LCAT has an anti-inflammatory effect in vivo. Discoveries in the past decade have shed light on the complex metabolic and antiatherosclerotic pathways of HDLs. These insights have fueled the development of HDL-targeted drugs. In particular, many efforts were devoted to the design of ApoA-I mimetic peptides mimicking the functionality of ApoA-I (Kruger et al., 2005; Navab et al., 2005; Buga et al., 2006; Peterson et al., 2007; Degoma and Rader, 2011). Our study suggests that it is feasible to design drugs that can enhance the physiological endogenous protective role of ApoA-I, which may have an application in inflammation-based cardiovascular diseases such as atherosclerosis.

**Fig. 8.** Schematic depiction of P2a mechanism of action. A, in physiological conditions, the HDL ApoA-I domain interacts with LCAT, causing an increase in the CE/C ratio. B, Hpt is an acute-phase protein, and its levels are increased during the inflammatory response. This leads to increased binding of Hpt to ApoA-I and, by interference with LCAT activity, to a reduction in CE/C ratio (reverse cholesterol transport). C, P2a has the same amino acid sequence of the binding site for ApoA-I. After the treatment in vivo, P2a displaces Hpt from ApoA-I, restoring the CE/C ratio.

**Authorship Contributions**

*Participated in research design:* Bucci, Cigliano, Abrescia, and Cirino.
*Conducted experiments:* Bucci, Cigliano, Vellecco, Rossi, Carlucci, and Ziaco.
*Contributed new reagents or analytic tools:* D’Andrea and Ziaco.
*Performed data analysis:* Cigliano, Vellecco, and Ianaro.
*Wrote or contributed to the writing of the manuscript:* Bucci, Cigliano, Sautebin, Abrescia, Pedone, Ianaro, and Cirino.

**References**


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