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Title Page

ApoA-I mimetic peptide P2a by restoring cholesterol esterification unmasks Apo A-I anti-inflammatory endogenous activity *in vivo*.

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ABBREVIATIONS: HDL, high density lipoproteins; ApoA-I, apolipoprotein A-I; LCAT, Lecithin-Cholesterol Acyl-Transferase; Hpt, Haptoglobin; CE, cholesteryl esters; C, cholesterol; PGE_2, prostaglandin E_2; COX, cyclooxygenase; NOS, nitric-oxide synthase.

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ABSTRACT

The acute phase protein Haptoglobin (Hpt) binds Apolipoprotein A-I (ApoA-I) and impairs its action on Lecithin-Cholesterol Acyl-Transferase (LCAT), an enzyme which plays a key role in the 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*. Aim of this study was to evaluate, whether P2a displaces Hpt from ApoA-I *in vivo*, and if this event leads to an anti-inflammatory activity. Mice received subplantar injection of carrageenan. Paw volume was measured before the injection and 2, 4, 6, 24, 48, 72 and 96h thereafter. In the same time points, concentrations of HDL-cholesterol (C) and cholesterol esters (CE) were measured by HPLC, while Hpt and ApoA-I plasma levels were evaluated by ELISA. Western blotting analysis for NOS and COX isoforms were also performed on paw homogenates. CE significantly decreased in carrageenan-treated mice during oedema development and negatively correlated with Hpt/ApoA-I ratio. P2a administration significantly restored the CE/C ratio. Contemporary, P2a displayed an anti-inflammatory effect on the late phase of oedema with a significant reduction in COX-2 expression coupled to an inhibition of PGE_2 synthesis, implying that, in presence of P2a, CE/C ratio rescue and oedema inhibition were strictly related. In conclusion, P2a effect is secondary to its binding to Hpt with consequent displacement of ApoA-I that is so able to exert an 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 inflammatory based diseases.

Introduction

Homeostasis of cholesterol (C) is essential for cell function and survival as it is toxic when 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 (HDL), apolipoprotein A-I (ApoA-I), apolipoprotein E, Lecithin-Cholesterol Acyl-Transferase (LCAT, E.C. 2.3.1.43) and several other enzymes (Rader, 2006; Rader et al., 2008a). Recently, several evidences have suggested a protective role for HDL in atherosclerosis based on their ability to promote the RCT (Rader, 2006). At the present stage, it is still unclear if part of the "protective" effect of HDL is due to functions beyond RCT and whether these functions could be enhanced or reduced following the interaction with circulating proteins. Indeed, it is known that HDL undergo to dramatic modification in structure and composition as a result of the concerted actions of the acute-phase response protein and inflammation (Khovidhunkit et al., 2005; Esteve et al., 2005). In these conditions, HDL particles progressively loose normal biological activities and acquire altered properties (Kontush and Chapman, 2006). For example the replacement of ApoA-I with serum amyloid A, occurring in small, dense HDL upon induction of the acute phase of inflammation, (Parks and Rudel, 1985; Coetzee et al., 1986) has been reported to have pro-atherogenic effects (Cabana et al.,1996; Lewis et al., 2004; O'Brien et al., 2005).

The critical role of inflammation at all stages of atherosclerosis is now recognized, including triggers, mediators and end-effectors (Libby, 2007; Kontush and Chapman, 2006). Recently, we have shown that the acute phase protein haptoglobin (Hpt) binds the HDL protein components ApoA-I and ApoE, impairing their key functions in RCT (Spagnuolo et al., 2005; Porta et al., 1999; Salvatore et al., 2007; Cigliano et al., 2009; Salvatore 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 in order to capture and

transport to the liver free haemoglobin (Langlois 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 (CE) addressing them to HDL for transport into the circulation (Rader et al., 2008b). 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 driving to: i) poor cholesterol removal from peripheral cells ii) low level of HDL cholesterol in the circulation (Spagnuolo et al., 2005; Salvatore et al., 2007; Cigliano et al., 2009). Moreover, several studies have confirmed that high levels of Hpt are associated to 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 amino acid sequence overlapping the stimulatory site for LCAT (P2a: Acetyl-LSPLGEEMRDRARAHVDALRTHLA-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 while, when incubated without Hpt, it did not affect LCAT cholesterol esterification (Spagnuolo et al., 2005). Since the anti-inflammatory activity of HDL and ApoA-I has been well documented (Kontush and Chapman, 2006; Rader, 2006; Sherman et al., 2010; Gomaraschi et al., 2008), we hypothesized that the ApoA-I mimetic peptide P2a could be also 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.

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Methods

Drugs and Reagents

Sheep anti-apo A-I and sheep anti-Hpt polyclonal antibody were purchased from SEROTEC [Oxford, UK]. [1α,2α-^{3}H]Cholesterol (45 Ci/mmole) was obtained from Perkin Elmer [Boston, MA, USA]. Sil-G plates for Thin Layer Chromatografy (TLC; thickness 0.25 mm) were obtained from Macherey-Nagel [Düren, Germany]. Chemicals of the highest purity, albumin serum bovine (BSA), cholesterol, cholesteryl linoleate, donkey anti-sheep IgG horseradish-linked (DAS-HRP), *o*-phenylenediamine, dextran sulfate (Dextralip 50, DS), carrageenan, molecular mass markers, Supelcosil LC-18 (5 μm particle size, 250 x 4.6 mm I.D.) were obtained from Sigma-Aldrich [St Louis, MO, USA]. Polystyrene 96 wells plates were purchased from Nunc [Roskilde, Denmark]. Bradford reagent was obtained from Bio-Rad (Bio-Rad laboratories, Segrate Milan, Italy). The antibodies against COX-2 and iNOS were from Transduction laboratories (U.S.A.). [^{3}H-PGE_2] was from Perkin-Elmer (Milan, Italy). All other reagents and compounds used were obtained from Sigma-Aldrich (Milan, Italy).

Peptides Synthesis

The peptide P2a (ApoA-I sequence from L141 to A164; acetyl-LSPLGEEMRDRARAHVDALRTHLA-amide) and the scramble peptide control (P2as: acetyl-RLSARLTLHEGPVALDEMRADRHA-amide) were synthesized on solid phase using PAL-PEG-PS resin (0.16 mmol/g) (Perspective Biosystem) by standard Fmoc (N-(9-Fluorenyl) methoxycarbonyl) chemistry. Amino acids coupling was performed using a 10 molar excess of Fmoc-amino acid, 9.9eq 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) and 20eq N,N-diisopropylethylamine (DIPEA). A solution of 30% piperidine in N,N-dimethylformamide (DMF)

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was used in the deprotection step (2x5min). Coupling reaction was performed for 60 min followed by a ten minutes acetylation step with acetic anydride (2M)/DIPEA (0.55M)/ HOBt (0.06M) in N-methylpirrolidone. Cleavage from the resin were achieved by treatment with trifluoracetic acid, triisopropyl silane, ethanedithiol, water, (94; 1; 2.5; 2.5 v/v/v/v) at room temperature for 3 hours. Peptides analysis and purification were performed by RP-HPLC on a C12 Proteo columns (Phenomenex) using a gradient of CH_3CN (0.1% TFA) in water (0.1% TFA) going from 20% to 50% in 30 min. Peptide identities were assessed by ESI mass spectrometry on Thermo Finnigan MSQ LC-MS (P2a: MW_{sperim} 2755.5 Da – MW_{calc} 2756.1 Da; P2as: MW_{sperim} 2755.8 Da – MW_{calc} 2756.1 Da).

Mouse paw oedema

Male Swiss mice (CD-1; Harlan, Italy) weighing 28-30 g were used for *in vivo* experiments. The experimental procedures were carried out 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 each group) and lightly anaesthetized with isoflurane. Each group of animals received subplantar injection of 50µl of carrageenan 1% (w/v) or 50µl of vehicle (saline) in the left hind paw. Paw volume was measured by using an hydropletismometer specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before the subplantar injection and 2, 4, 6, 24, 48, 72 and 96h 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 oedema. Each group of animals received i.p.(100µl) administration of P2a peptide (0.3, 0.6, 1 mg/kg), P2a scramble peptide (P2as,

1mg/kg), or vehicle (saline). All peptides were administrated immediately before the injection of carrageenan and 24h thereafter.

ApoA-I and Hpt immunoassay

The concentration of ApoA-I and Hpt was determined by ELISA using mouse antigens as standards for calibration, isolated as previously reported (Spagnuolo et al., 2003) and exhibiting over 98% purity by electrophoresis and densitometry analysis,. Aliquots of plasma (50 µl from 1:1000, 1:10000, 1:20000, 1:45000, 1:60000, and 1:100000 dilutions) were diluted in coating buffer (7.3 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6), loaded into the wells of a microtiter plate, and processed essentially according to a published procedure (Cigliano et al., 2005). In particular, sheep IgG (anti-ApoA-I or anti-Hpt respectively) was used as primary antibody, and DAS-HRP IgG as secondary antibody for colour development. Measures were done using a calibration curve, obtained by determining the immunoreactivity of 1, 0.5, 0.25, 0.125, 0.065, 0.033, 0.016 ng of standard Hpt protein and 0.4, 0.24, 0.16, 0.08, 0.04, 0.016 ng of standard ApoA-I protein.

LCAT activity assay

Plasma samples from treated and control animals were treated with 50 mM $MnCl_2$ and 0.1% dextran sulfate (50 kDa) according to a published procedure to remove VLDL and LDL (Burstein et al., 1970). The LCAT activity *in vitro* was measured using proteoliposomes (ApoA-I/lecithin/cholesterol molar ratio of 1.5:200:18) as substrate as previously reported (Spagnuolo et al., 2005; Cigliano et al., 2009). The enzyme activity was expressed as enzyme units (nmoles of cholesterol esterified per hour per millilitre of plasma).

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Titration of cholesterol and cholesteryl esters in HDL

The ratio of CE with unesterified C, in HDL, was assumed to reflect the LCAT activity in the plasma (Cigliano et al, 2005). Plasma samples of treated or control animals were treated with 50 mM $MnCl_2$ and 0.1% dextran sulfate (50 kDa) according to a published procedure to remove VLDL and LDL (Burstein et al., 1970). After this treatment, two 25 µl aliquots were used for measuring the amounts of unesterified and total C. One aliquot was incubated (1h; 75 °C) with 0.25 ml of ethanol, while the other one with 0.25 ml of ethanol containing 5 M KOH. After incubation, both mixtures were supplemented with 0.15 ml of 1% NaCl and, after addition of 2 ml of ice-cold hexane, vigorously shaken for 2 min. The hexane extract was taken on and the lower phase was likewise extracted two more times. The three extracts were pooled and dried under nitrogen stream. The residue was dissolved in 0.2 ml of acetonitrile/isopropanol (57:43, v:v), and 20 µl were processed by reverse-phase HPLC. The chromatography was performed by a C18 column at 40°C with 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 used to calculate the amount of CE as "total minus unesterified cholesterol". Calibration curves ($r^2 \geq 0.9997$), obtained by injecting different amounts (n= 12) of standard C, were used for quantitative analysis.

Western blot analysis

Paws from different groups of mice were harvested after 24 and 48h from carrageenan or vehicle injection, and homogenized in modified RIPA buffer (Tris HCl 50mM, pH 7.4, triton 1%, Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, phenylmethanesulphonylfluoride 1 mM, aprotinin 10µg/ml, leupeptin 20mM, NaF 50 mM) using a polytron homogenizer (two cycles of 10 sec at maximum speed) on ice. After centrifugation at 12,000 rpm for 15 min, protein concentration

was determined by Bradford assay using BSA as standard (Bio-Rad Laboratories, Milan, Italy). 40 µg of the denatured proteins were separated on 10% SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in PBS-tween 20 (0.1 %, v/v) containing 5% non fat dry milk and 0.1 % BSA for 1 hour at room temperature, and then incubated with anti-COX2 (1:1000), anti-iNOS (1:1000) overnight at 4°C. The filters were washed with PBS-tween 20 (0.1%, v/v) extensively for 30 min, before incubation, for 2 hours at 4°C, with the secondary antibody (1:5000) conjugated with horseradish peroxidase antimouse IgG. The membranes were then washed and immunoreactive bands were visualized using an Enhanced Chemiluminescence Substrate (ECL; Amersham Pharmacia Biotech, San Diego, CA, USA).

PGE_2 exudates levels

Mice from different groups were euthanized after 24 and 48h from carrageenan or vehicle injection. Paws were cut and centrifuged at 4000 rpm for 30 min. Exudates (supernatants) were collected with 100µl of saline and used for PGE_2 quantification. To determine PGE_2 levels, proteins were removed from the exudates with $ZnSO_4$ 30% for 15 min (Thomsen et al., 1990). PGE_2 were determined in de-proteinized exudates by radioimmunoassay according to manufactures instructions.

Statistical analysis

Result were expressed as mean ± s.e.m. or as mean± SD Statistical analysis was determined by ANOVA followed by Bonferroni's 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 was less than 0.05. Each sample was processed at least in triplicate.

Results

Cholesterol esterification is reduced in mouse oedema

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

In the early phase of carrageenan-induced inflammation (0-6 h), no differences were found in the CE/C ratio compared to the vehicle (data not shown). In the late phase of inflammation (24-48h), the values of the CE/C ratio were found significantly decreased in carrageenan-treated mice (21.5 ± 1 at time 0 versus 12 ± 0.6 at 24h, $P < 0.01$; 21.5± 1 at time 0 versus 10.5 ± 0.9 at 48h, $P < 0.01$, figure 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 stimulate the enzyme LCAT, were found 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, following carrageenan injection, showed a biphasic trend: an early increase peaking 2h after the carrageenan injection (29.1 ± 1.5 in inflamed mice versus 14.1 ± 0.5 μM in controls; $P<0.01$), and a second late response peaking 48h after (66.6 ± 4 in inflamed mice versus 13.8 ± 2.8 μM in controls; $P<0.01$), returning to the physiological values at 144h (14.2 ± 3 in inflamed mice versus 13.4 ± 2.1 μM in controls). The Hpt/ApoA-I ratio was found negatively

correlated with the CE/C ratio ($P<0.01$; $R=0.94$, Fig. 3). These data well fit with an inhibitory role of Hpt in the ApoA-I-dependent activity of cholesterol esterification on HDL.

P2a peptide inhibits mouse paw oedema in a dose-dependent manner

In the early phase of carrageenan-induced paw oedema (0-6 h), no differences were found among groups treated with P2a at all doses tested compared to the vehicle (Fig. 4). In the late phase of oedema (24-96 h), injection of P2a caused a significant and dose-dependent inhibition of oedema (Fig. 4). The specificity of P2a effect was confirmed by the finding that the scrambled peptide (P2as) did not affect the oedema development in both phases (Fig. 4).

P2a peptide restores cholesterol esterification

It is known that the binding of Hpt to ApoA-I is associated to a 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). In order to evaluate whether the P2a peptide displaces Hpt *in vivo*, different doses of P2a (0.3, 0.6, 1 mg/kg, i.p.), or of a scrambled peptide (P2as), were injected in mice. When mice were treated with 0.6 or 1 mg/kg of P2a the CE/C ratio was found significantly restored (Fig.5A). No effect of P2a on the HDL total C (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 PGE_2 generation

P2a exerted its anti-inflammatory action in the second phase of carrageenan-induced paw oedema. In order to identify the molecular mechanism/s responsible for this anti-inflammatory effect, the expression of inducible isoforms of nitric oxide synthase and cyclooxygenase COX, i.e. iNOS and COX2, were evaluated at 24 and 48h following carrageenan administration. As shown in figure 6,

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western blot analysis did not reveal any significant alteration in COX2 expression at 24h from oedema induction, in all three groups of mice treated with P2a. Conversely, after 48h from carrageenan injection, P2a (1mg/kg) significantly reduced COX2 expression (Fig. 6). The COX2 involvement was also confirmed by the significant reduction of PGE_2 levels in paws exudates obtained from mice treated with P2a (Fig. 7). P2a administration did not affect the iNOS and eNOS expression at all time points (supplemental figure).

Discussion

It is known that the binding of Hpt to ApoA-I is associated to a 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; Cigliano et al., 2009; Salvatore 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 is still not determined if this mechanism is relevant *in vivo*. In order to address this issue we tested if P2a could exert a similar activity *in vivo*. By monitoring plasma CE/C ratio and Hpt levels during the oedema development, we found an inverse correlation between CE/C and Hpt/ApoA-I ratios. The inflammatory response, induced by carrageenan in mice, is coupled to 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 esters production confirming the physio-pathological relevance of this mechanism in inflammation

On the late phase of mouse paw oedema 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, the anti-inflammatory activity of P2a should rely on different mechanism(s). We found that Hpt levels were elevated of about six fold in the late phase of this inflammatory model. Thus, we hypothesise that Hpt, by binding ApoA-I, impairs LCAT function. In other words, as summarized for clarity in figure 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 P2a effect was confirmed by the experiments performed using a scrambled peptide, where the mismatching of

amino acids position, 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 oedema (24-96h). It is well know that in this mouse model of oedema are involved, at different time points, COX2 derived eicosanoids and/or iNOS or eNOS derived NO (Posadas et al., 2004). P2a administration did not modulate eNOS expression. These findings well correlate with the lack of activity of P2a in the early phase of the oedema. In the late phase, where P2a significantly modifies the cholesterol biochemical pathway, there was also a reduction in COX2 expression as well as of PGE_2 levels. This finding implies that the two phenomena observed in presence of P2a, i.e. CE/C ratio rescue and oedema inhibition, are strictly 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, P2a anti-inflammatory effect is evident at the same time point in which recovering of CE/C ratio occurs i.e. 48h following carrageenan injection. This hypothesis is further supported by the fact that P2a significantly inhibits exclusively the second phase of the oedema where Hpt level reaches its maximum.

In conclusion we have demonstrated that P2a, an ApoA-I derived peptide with 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 anti-atherosclerotic pathways of HDL. 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 (Degoma et al.; 2011; Navab et al., 2005; Kruger et al., 2005; Peterson et al., 2007; Buga et al., 2006). Our study suggest that it is feasible to design drugs that can enhance the physiological endogenous protective role of ApoA-I which may find an application in those inflammatory based cardiovascular diseases such as atherosclerosis.

Authorship contributions

Participated in research design: Bucci, Cigliano, Abrescia and Cirino

Conducted experiments: Bucci, Cigliano, Vellecco, Rossi, Carlucci and Ziaco

Contributed new reagents: D'Andrea and Ziaco

Performed data analysis: Ianaro, Cigliano and Vellecco

Wrote or contributed to the writing of the manuscript: Bucci, Cigliano, Abrescia, Pedone, Sautebin, Ianaro and Cirino

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Footnotes

* the authors equally contributed to the paper

Legends for Figures

Fig. 1. C and CE ratio in HDL isolated from mice plasma. Groups of three animals were sacrificed at 2, 4, 6, 24, 48, 72, 96, and 144 h after injection of carrageenan. The control group received vehicle only. Blood was collected and the HDL fraction was isolated from plasma of carrageenan treated mice and analyzed for C and CE content by HPLC. The molar concentrations of C and CE in the HDL were determined and the molar ratio CE/C was calculated. The samples were analysed in triplicate and the data are expressed as mean ± standard deviation.

Fig. 2. Titration of Hpt in plasma from mice treated with carrageenan. Groups of three mice were sacrificed at 2, 4, 6, 24, 48, 72, 96, and 144 h following carrageenan injection. The control values are reported at time 0 in the graph. Blood was collected, and plasma from each animal was prepared. Samples were analyzed by ELISA for measuring the concentration of Hpt (filled circles) and ApoA-I (open squares). The data are expressed as means ± standard deviation, n=3 separate experiments.

Fig. 3. Correlation between Hpt/ApoA-I ratio and CE/C ratio. Plasma was obtained from carrageenan treated mice and control mice at different timepoints. The plasma levels of Hpt and ApoA-I were measured by ELISA, and the molar concentrations of C and CE in the HDL were analysed by HPLC. Hpt/ApoA-I and CE/C ratios were calculated from triplicates. The Hpt/ApoA-I averages were plotted versus the homologous CE/C averages.

Fig. 4. Effect of P2a peptide on carrageenan-induced paw oedema. P2a (0.3, 0.6, 1 mg/kg), P2as (1 mg/kg) or vehicle were administrated i.p. immediately before the subplantar injection of carrageenan (50 µl) and 24h thereafter. Data are expressed as mean±s.e.m. n=6 for each group of treatment, $p<0.01$; * = $p<0.05$ versus vehicle.

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Fig. 5. (A) Effect of P2a on Hpt inhibition in cholesterol esterification**.** Blood was collected by intracardiac puncture from mice treated with P2a (0.3, 0.6, 1 mg/kg, i.p.), or P2as (1 mg/kg, i.p.) 24h or 48h after carrageenan injection. The HDL fraction was isolated from each plasma, and analyzed for C and CE content by HPLC. Data are expressed as CE/C ratio, it is notably that the increase of CE/C ratio was strictly related to P2a anti-inflammatory effect. as described in figure 8 panel C. The samples were analysed in triplicate and the data are expressed as mean ± standard deviation. For each group of treatment n=6, **=$p<0.001$; * = $p<0.01$ versus treated mice 24h or 48h after carrageenan injection ; + = significantly different from control mice. (B) Effect of P2a on the HDL total C (HDL-C). The molar concentrations of C and CE in the HDL were determined as reported in figure 5A and the total C was calculated as C+CE. The samples were analysed in triplicate and the data are expressed as mean ± standard deviation.

Fig. 6. Effect of P2a administration on COX2 expression in inflamed paws. After 24 and 48h from carrageenan injection, mice paws harvested from different groups of treatment were homogenated and western blot analysis for COX2 was performed. A) Representative western blot for COX2. S = sham, V = vehicle, P2as = P2a scramble peptide, P2a 0.6 = P2a 0.6 mg/kg, P2a 1 = P2a 1 mg/kg B) western blot densitometric analysis for COX2 (n=3 experiments) Data are expressed as mean±s.e.m. .

Fig. 7. Effect of P2a on PGE_2 levels in inflamed paws. After 24 and 48h from carrageenan injection, mouse paws harvested from different groups of treatment were centrifuged, exudates collected and used for PGE_2 determination. Data are expressed as mean±s.e.m. For each group of treatment n=4. ** = $p<0.01$ and ***= $p<0.001$ versus sham; ° = $p<0.05$ and °° = $p<0.01$ versus vehicle

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Fig. 8. Schematic depiction of P2a mechanism of action.

Panel A: In physiological conditions HDL ApoA-I domain interacts with LCAT causing an in increase in CE/C ratio.

Panel B: Hpt is an acute phase protein and its levels are increased during the inflammatory response. This brings to an increased binding of Hpt to ApoA-I and, by interfering with LCAT activity, to a reduction in CE/C ratio (reverse cholesterol transport).

Panel C: P2a has the same aminoacidic sequence of the binding site for ApoA-I. Following the treatment *in vivo,* P2a displaces Hpt from ApoA-I restoring the CE/C ratio.

Abbreviations: HDL = high density lipoproteins; ApoA-I = apolipoprotein A-I; LCAT = Lecithin-Cholesterol Acyl-Transferase; Hpt = Haptoglobin; CE = cholesteryl esters; C = cholesterol

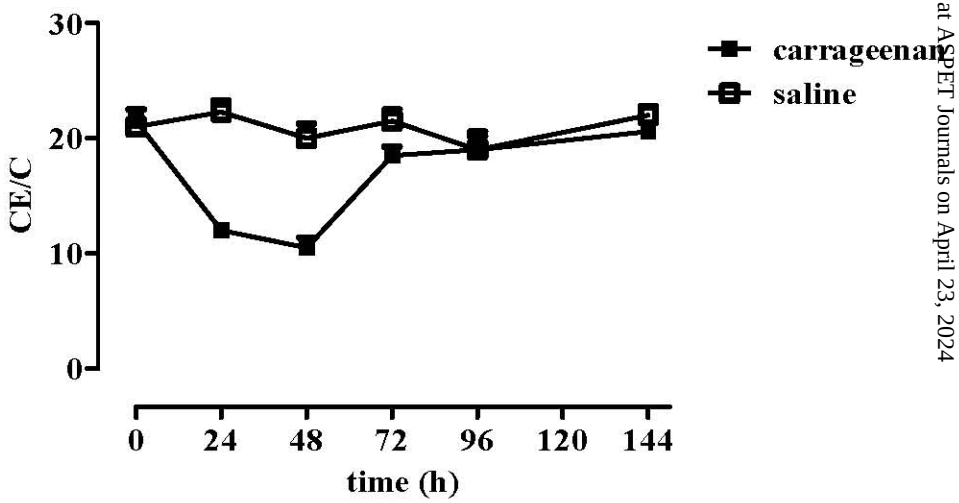


Figure 1

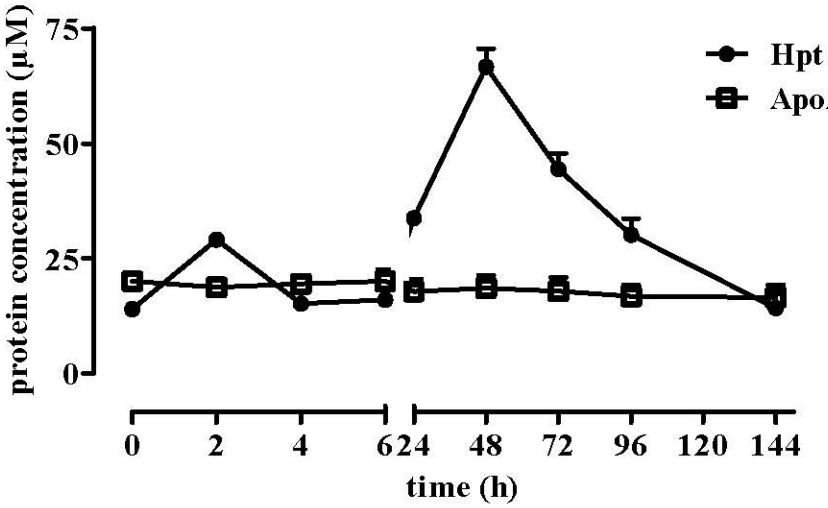
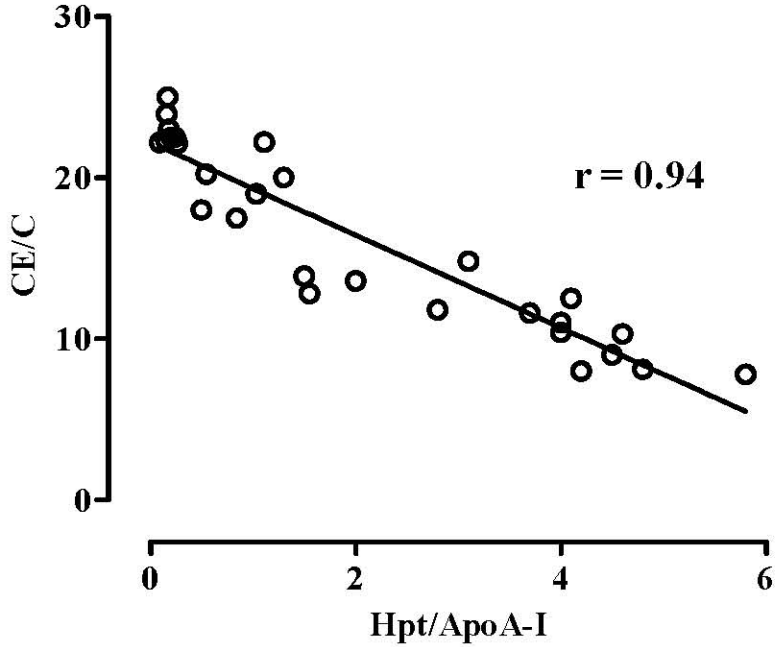


Figure 2



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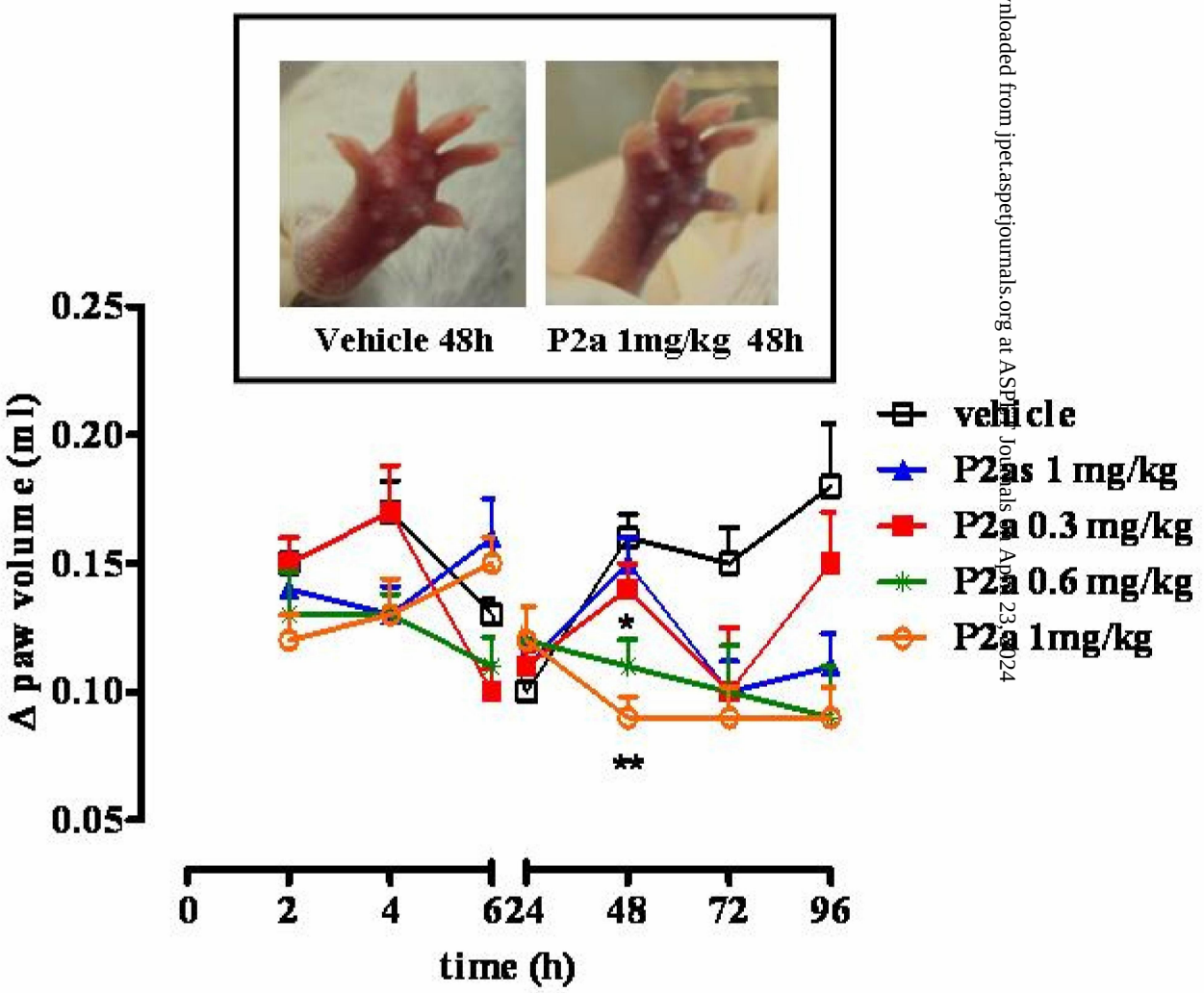

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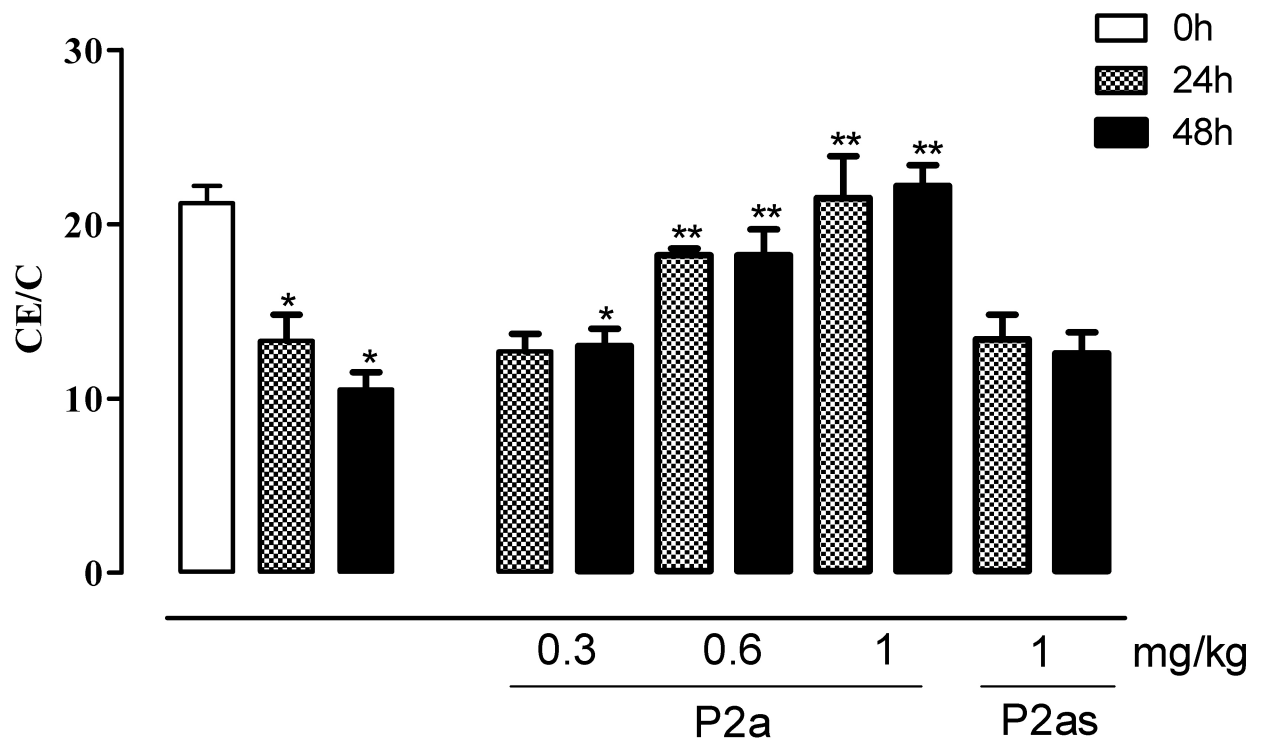
0h
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48h
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30
20
10
0
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0.3
0.6
1
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mg/kg
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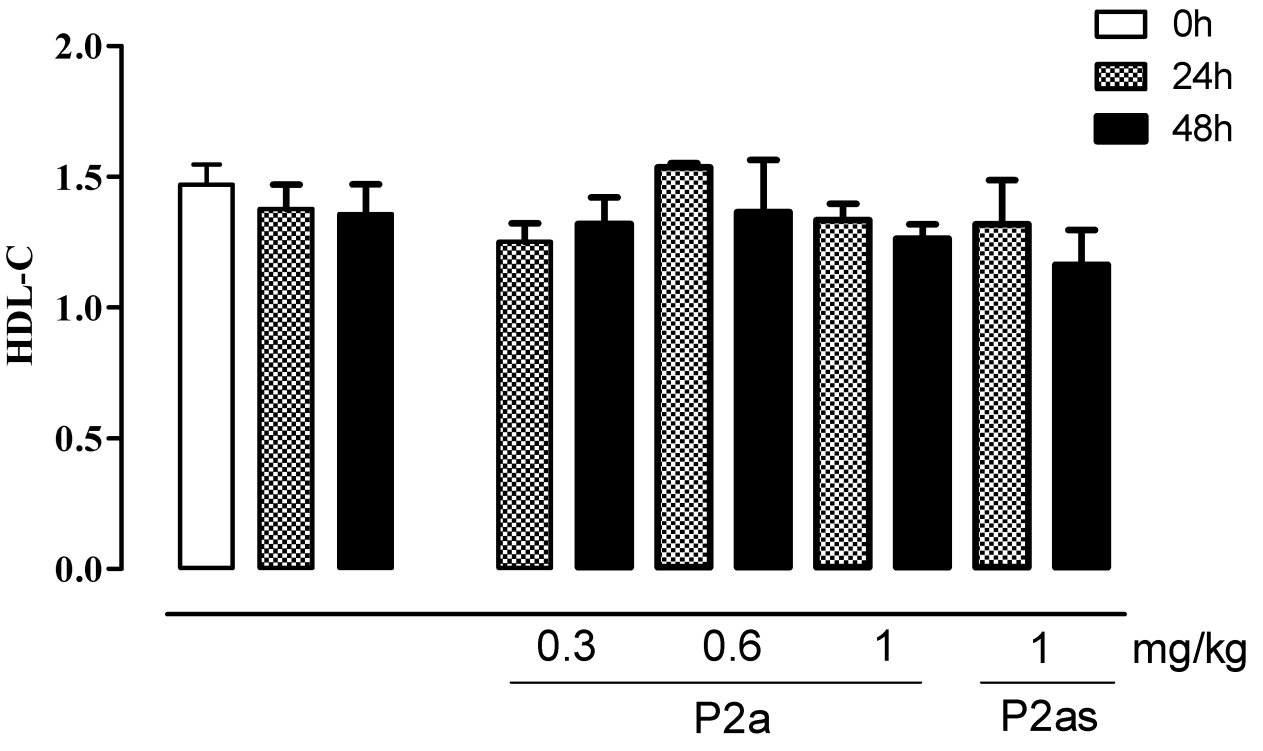
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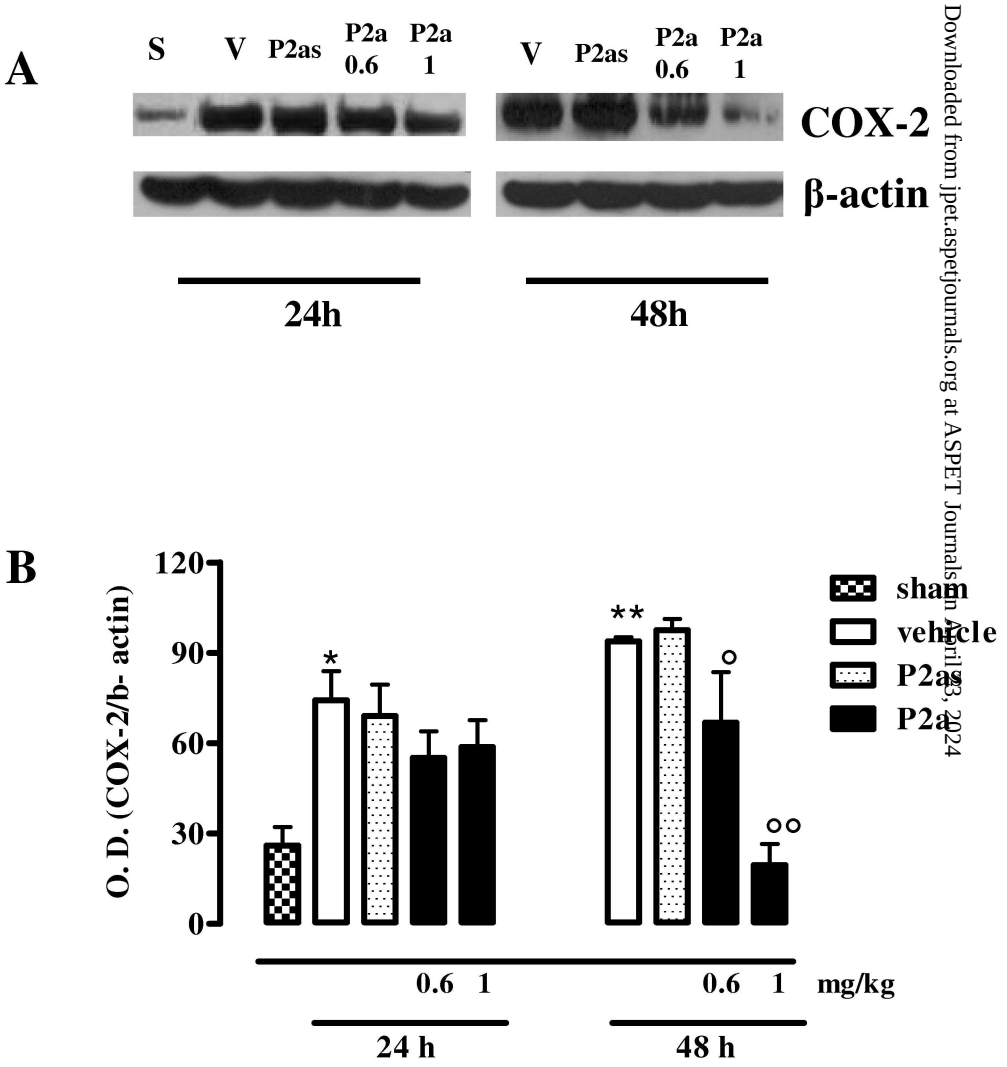

Figure 6

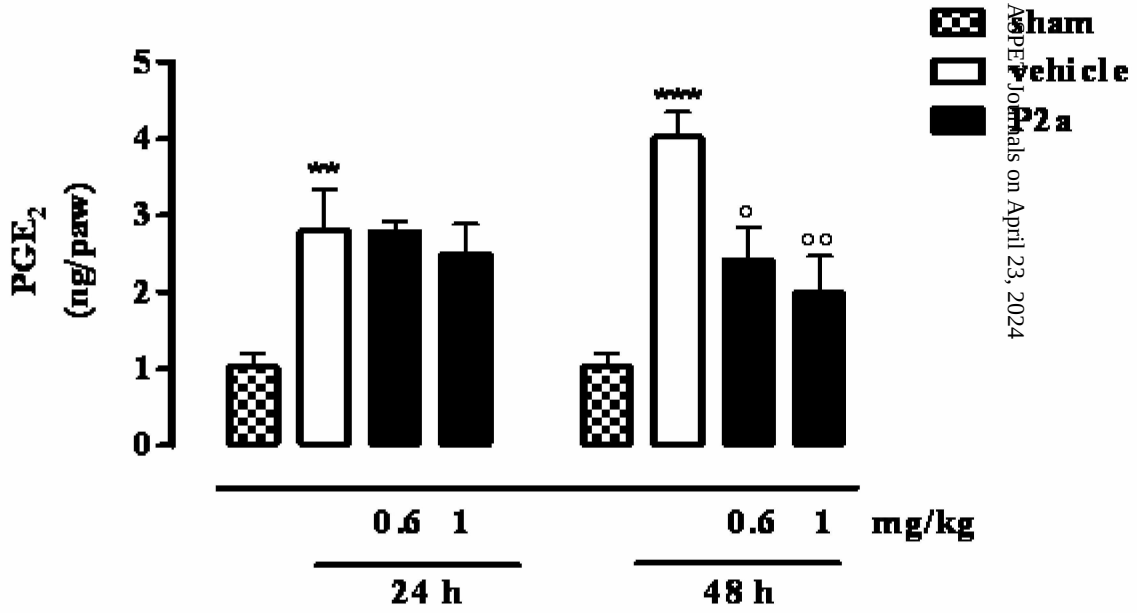

Figure 7

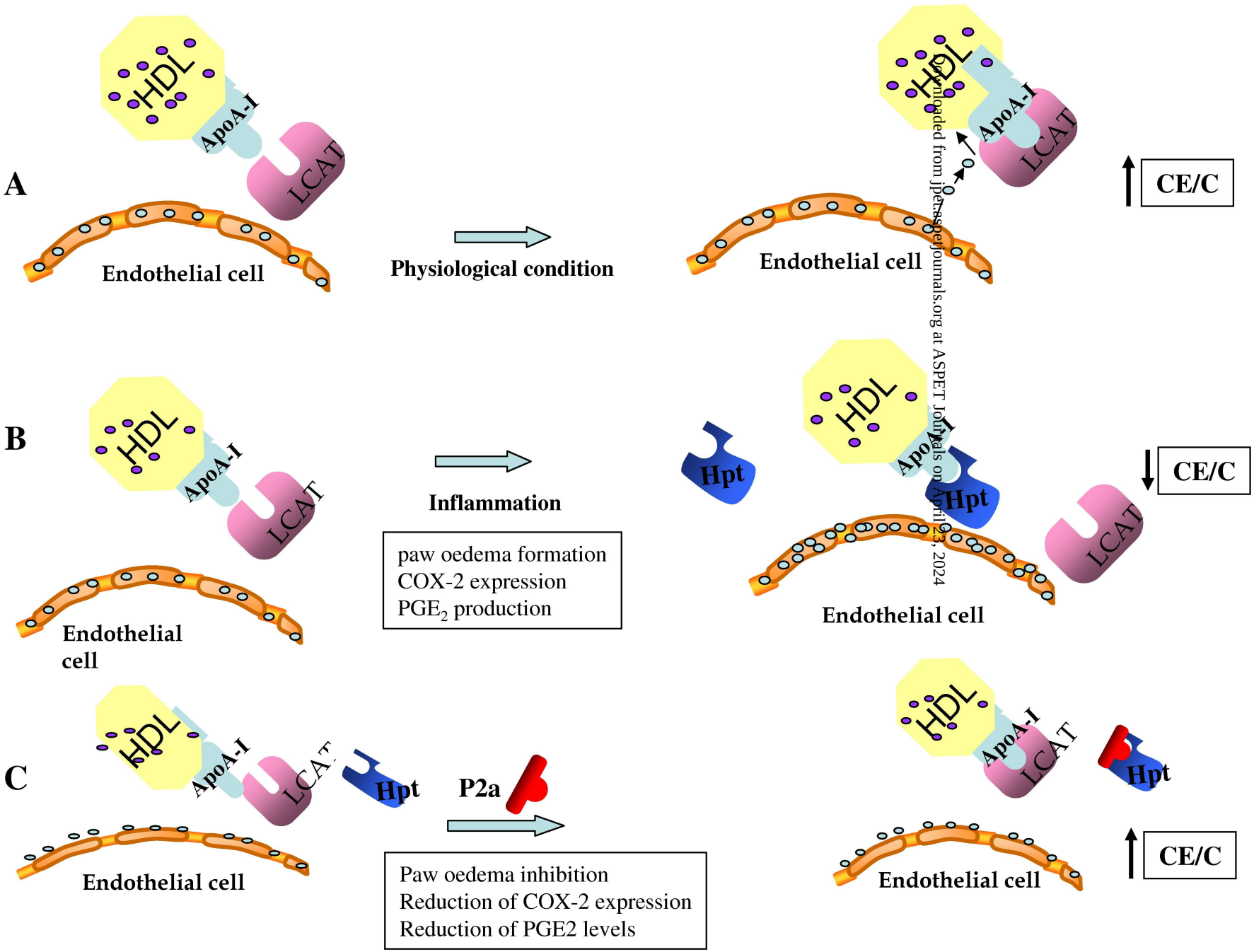

Figure 8