Immune-regulation of the apolipoprotein *A-I/C-III/A-IV* gene cluster in experimental inflammation

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Abstract

Apolipoprotein A-IV is a member of the *apo A-I/C-III/A-IV* gene cluster. In order to investigate its hypothetical coordinated regulation, an acute phase was induced in pigs by turpentine oil injection. The hepatic expression of the gene cluster as well as the plasma levels of apolipoproteins were monitored at different time periods. Furthermore, the involvement of the inflammatory mediators' interleukins 1 and 6 and tumor necrosis factor in the regulation of this gene cluster was tested in cultured pig hepatocytes, incubated with those mediators and *apo A-I/C-III/A-IV* gene cluster expression at the mRNA level was measured. In response to turpentine oil-induced inflammation, a decreased hepatic *apo A-IV* mRNA expression was observed (independent of *apo A-I* and *apo C-III* mRNA) not correlating with the plasma protein levels. The distribution of plasma apo A-IV experienced a shift from HDL to larger particles. In contrast, the changes in *apo A-I* and *apo C-III* mRNA were reflected in their corresponding plasma levels. Addition of cytokines to cultured pig hepatocytes also decreased *apo A-IV* and *apo A-I* mRNA levels. All these results show that the down-regulation of apolipoprotein *A-I* and *A-IV* messages in the liver may be mediated by interleukin 6 and TNF-α. The well-known HDL decrease found in many different acute-phase responses also appears in the pig due to the decreased expression of apolipoprotein A-I and the enlargement of the apolipoprotein A-IV-containing HDL.
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1. Introduction

Reduced plasma high density lipoprotein (HDL) is a well-established risk factor for the development of atherosclerosis. Although the mechanisms responsible for reduced HDL levels are complex [1], apolipoprotein components play an important role. This role is better known for apolipoprotein A-I than for other apos which constitute these particles. In this regard, the role of apolipoprotein A-IV (apo A-IV) in determining HDL concentration and influencing the inflammatory processes is being actively investigated. It has been shown that overexpression of the human apo A-IV [2] in transgenic mice has a protective effect against the formation of diet-induced aortic lesions and inhibits experimental colitis [3].

Human apo A-IV is synthesized in the small intestine and the liver [4]. Apo A-IV is found mainly associated with high density lipoproteins [5,6] in plasma. The role of apo A-IV in lipoprotein metabolism has not been fully defined [7]. It has been proposed that apo A-IV is involved in the metabolism of triglyceride (TG)-rich lipoproteins, possibly by modulating the activation of lipoprotein lipase in the presence of apo C-II [8]. Experiments with transgenic mice suggest that this protein regulates TG transport and cholesterol levels [9]. Apo A-IV is also considered to play a role in reverse cholesterol transport by stimulating lecithin:cholesterol acyltransferase activity. Apo A-IV associates with HDL upon the action of the lecithin:cholesterol acyltransferase, in a reaction that causes depletion of HDL surface lipids and stabilizes HDL [10,11]. Moreover, apo A-IV participates in HDL particle conversion produced by cholesteryl ester transfer protein [12].

Apo A-IV is a member of a closely linked, gene cluster localized in human and pig chromosomes 11 and 9, respectively, which also includes apolipoproteins A-I and C-III [13,14]. Common enhancers regulating *apo C-III* and *apo A-IV* expressions have been previously described [15]. These facts point to a coordinate regulation of this *A-I/C-III/A-IV* gene cluster in response to different stimuli in vivo, a hypothesis that requires testing in different experimental settings and species.

Following an acute injury, alterations of lipoprotein concentrations have been reported in several animal models [16–20]. The decrease in HDL with the concomitant diminution of apo A-I and apo A-IV [21,22] has been described as a finding related to the acute-phase response. It has been proposed that this decrease is induced by an accelerated catabolism of these particles as corroborated by the overexpression of lipid associated phospholipase A_2 [23]. Despite this finding, an increase in *apo A-IV* expression noted in double knock-out mice for apo E and γ-interferon suggests [24] that the acute phase-decrease in HDL may also be induced by decreased expressions of main apolipoprotein components such as apo A-I and apo A-IV. This in turn indicates a coordinate regulation of the *A-I/C-III/A-IV* gene cluster in the liver. To address this hypothesis, acute phase was induced by turpentine oil injection in pigs and the hepatic expression of the *A-I/C-III/A-IV* gene cluster was monitored at different time periods. Inflammation may be regulated by soluble mediators such as interleukins (IL-1 and IL-6) and tumor necrosis factor (TNF-α), considered major inducers of the acute-phase protein response [25]. To demonstrate such involvement in the regulation of the gene cluster in this animal model, pig hepatocytes were incubated with these mediators and the mRNA expression was measured.

2. Results

2.1. Effect of turpentine administration on plasma lipids, lipoproteins and apolipoproteins

Table 1 shows the concentration of pig plasma lipids at different times after turpentine administration. No statistically significant changes in plasma total cholesterol concentration following the administration of this agent were observed. Time course distribution of cholesterol in different lipoproteins of pig plasma from a representative animal after turpentine administration is shown in Fig. 1. Quantitative data of all animals are shown in Table 1. No significant change in VLDL cholesterol was observed. During the course of turpentine administration, there was a progressive increase in LDL concentration along with a parallel decrease in HDL cholesterol (Fig. 1B and C). These changes only showed statistical significance 48 h after the treatment. The uniformity of HDL peak size was preserved under the influence of this inflammatory agent.

Triglyceride levels showed a significant increase 8 h after the treatment, and recovered control values after 48 h. The distribution of these compounds in plasma lipoproteins is shown in Fig. 1 and corresponds to a representative animal. In control animals (Fig. 1A), most of the triglycerides were carried by VLDL and those remaining were in LDL particles. After turpentine administration, the transient peak in total triglycerides observed after 8 h (Table 1) was mainly due to an increase in content of TG in VLDL particles (Fig. 1B). At 48 h, despite no change in total content of TG, a dramatic redistribution of these compounds was observed. The increase in a subgroup of LDL-like particles was particularly notable.

Concentration and distribution of plasma apo A-I, C-III and A-IV in the lipoproteins are shown in Table 2. Plasma levels of apolipoprotein A-I progressively decreased reaching a significant value at 48 h after

Table 1
Plasma lipids of pigs after turpentine oil injection

	Control (n = 10)	Hours post-injection	
		8 h (n = 8)	48 h (n = 9)
Total cholesterol (mmol/l)	2.3 ± 0.6	2.6 ± 0.5	2.3 ± 0.6
Triglycerides (mmol/l)	0.4 ± 0.2	0.8 ± 0.2[a]	0.4 ± 0.3[b]
VLDL-C (mmol/l)	0.1 ± 0.06	0.1 ± 0.01	0.1 ± 0.07
LDL-C (mmol/l)	1.2 ± 0.2	1.4 ± 0.3	2.0 ± 0.2[A,B]
HDL-C (mmol/l)	1.1 ± 0.1	1.1 ± 0.3	0.5 ± 0.1[A,B]

Values are mean ± standard deviation of the number of animals indicated in brackets. Statistical analysis was carried out using one-way ANOVA followed by Tukey–Kramer post hoc test ([A]$P < 0.001$ and [a]$P < 0.05$ vs control and [B]$P < 0.001$ and [b]$P < 0.05$ vs 8 h).
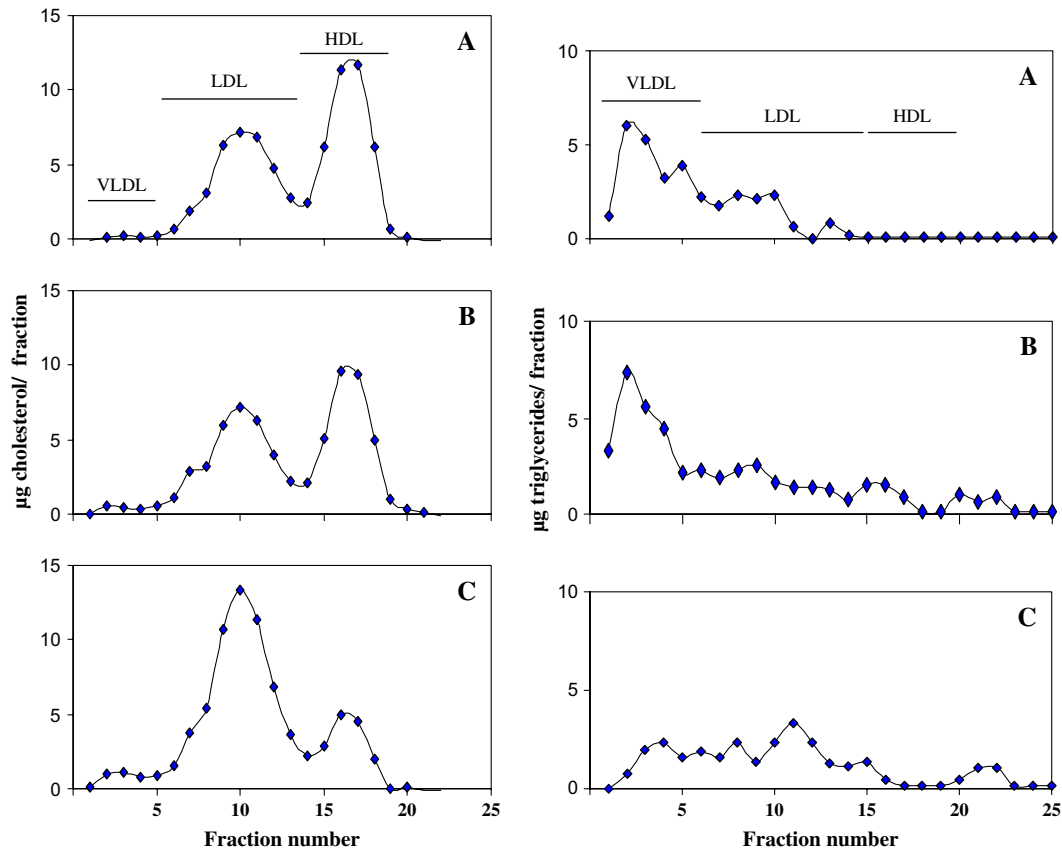
Fig. 1. Plasma lipoprotein distribution of cholesterol and triglycerides in pigs subject to induced experimental inflammation. Plasma samples were separated by Superose 6B FPLC column chromatography and fractions analyzed for cholesterol and triglycerides. Results are shown as micrograms of cholesterol or TG per fraction. (A) Control pigs; (B) 8 h after turpentine administration; and (C) 48 h after turpentine administration.

Table 2
Apolipoprotein concentration and distribution in different plasma lipid fractions in pigs

	Control ($n = 10$)	Hours post-injection	
		8 h ($n = 8$)	48 h ($n = 9$)
Total apo A-I (mg/ml)	3 ± 0.4	2.6 ± 0.2	1.2 ± 0.2[a,b]
Plasma distribution of apo A-I (%)			
LDL	4.2 ± 1.0	4.2 ± 0.9	5.7 ± 2.6
HDL	95 ± 1.0	94 ± 3.2	93 ± 2.6
Lipoprotein-free	1.0 ± 0.4	0.9 ± 0.3	1.5 ± 0.3
Total apo A-IV (arbitrary units)	32 ± 5	33 ± 5	31 ± 4
Plasma distribution of apo A-IV (%)			
LDL	1.5 ± 0.6	2.4 ± 0.8	4.2 ± 2[a]
HDL	91.5 ± 2.0	92.9 ± 2.6	89.4 ± 2[b]
Lipoprotein-free	7 ± 1.8	4.7 ± 1.5[a]	6.4 ± 2
Total apo C-III (arbitrary units)	0.35 ± 0.06	0.4 ± 0.07[a]	0.32 ± 0.05[b]

Values are mean ± standard deviation of the number of animals indicated in brackets. Statistical analysis was carried out using one-way ANOVA followed by Tukey−Kramer post hoc test ([a]$P < 0.001$ vs control and [b]$P < 0.001$ vs 8 h).

turpentine administration. No significant change in lipoprotein distribution was observed, HDL being the main (94%) lipoprotein containing apo A-I. This fact, together with the high association between apo A-I and HDL-cholesterol levels ($r = 0.9577$, $P < 0.0001$) are indicating that the decrease in apolipoprotein A-I was responsible for the lower HDL-cholesterol levels observed after the administration of turpentine.

No change in plasma apo A-IV concentration was observed after turpentine administration. However, distribution of apo A-IV in plasma underwent important changes in the course of time after turpentine administration. Western-blot analysis of a representative animal is displayed in Fig. 2A and quantitative data of all animals are shown in Table 2. A trend in the increase of LDL, that reached statistical significance at 48 h, was noted. This change was due to the decrease in lipoprotein-free apolipoprotein and in HDL at 8 and 48 h, respectively, after turpentine administration.

To estimate plasma apo C-III concentration, commercially available antibodies were tested. While the rabbit anti-human antibody did not react (data not shown), the rabbit anti-mouse antibody had a cross-reaction with the pig plasma protein, as evidenced by
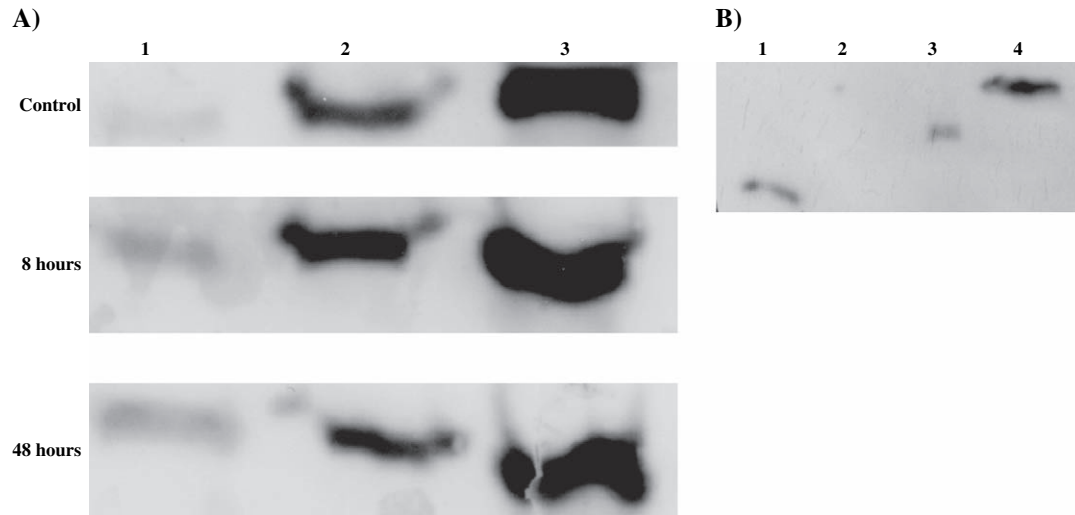
A)

B)

Fig. 2. Western-blot analysis of apolipoproteins A-IV and C-III. Apolipoprotein A-IV detection in: (A): 5%, 0.5% and 20% of total volume of LDL (lane 1), HDL (lane 2) and lipoprotein-free (lane 3) fractions, respectively, were loaded in the different lanes. Panel showing the specificity of anti-mouse apo C-III antibody (B). Two microlitres of mouse and human plasma diluted 1/20 were loaded in lanes 1 and 2, respectively; while in lanes 3 and 4, 2 μl of pig plasma and 60 μg of hepatic total protein were electrophoresed. Primary antibody was used at a 1/1000 dilution and secondary antibodies at a 1/15 000 dilution.

Western-blot analysis (Fig. 2B, lane 3). This antibody specifically recognized the mouse protein (lane 1), as expected; but not the human protein (lane 2). To detect the pig apo C-III, a 20-fold higher concentration of plasma than in the case of mouse had to be employed. Despite this fact, the intensity of the band was quite faint (lane 3). The analysis of hepatic concentrations in pig and mouse apo C-III evidenced a good recognition of protein by this antibody (data not shown). From the quantitative data from Fig. 2B it can be inferred that plasma pig apo C-III concentration was 50-fold lower (in the range of 50 μg/dl) than the mouse and that its molecular weight was higher than the mouse protein, suggesting a post-translational modification. The comparison of sizes between lanes 3 and 4, corresponding to porcine plasma and hepatic apo C-III, respectively, confirmed that pig apo C-III underwent the described proteolytic processing to remove the signal peptide.

With the information gathered above, a new ELISA was set up to estimate plasma concentration of apo C-III and its distribution among lipoproteins. Plasma apo C-III levels (Table 2) showed a transitional significant increase 8 h after the turpentine treatment, and returned to control values at 48 h in concordance with the trend observed for plasma TG. VLDL was the only lipoprotein carrying apo C-III and no change in distribution was observed by the treatment.

2.2. Effect of turpentine administration on plasma levels of inflammatory markers

Table 3 shows the concentration of plasma TNF-α, IL-1β, IL-6 and CRP levels at different sampling times after turpentine administration. No significant change was observed in the levels of TNF-α. In contrast, IL-1β and IL-6 levels increased by the toxic administration although statistical significance was not observed until 48 h after the treatment. Similar behaviour was observed for C-reactive protein. These results indicate that an inflammatory status is openly induced after 48 h of turpentine administration in pigs.

In order to establish an association among plasma inflammatory markers and lipid parameters, a correlation analysis was carried out and the results are shown in Table 4. No significant association was found between TNF-α levels and lipid parameters. However, IL-1β and IL-6 levels were directly correlated with LDL-cholesterol and C-reactive protein and inversely with HDL-cholesterol and apo A-I levels. Likewise, C-reactive protein levels inversely correlated with HDL-cholesterol and apo A-I concentrations. The high

Table 3

Plasma levels of inflammatory markers in pigs after turpentine oil injection

Inflammatory markers		Hours post-injection	
	Control ($n = 10$)	8 h ($n = 8$)	48 h ($n = 9$)
TNFα (pg/ml)	71 ± 17	86 ± 9	84 ± 14
IL-1β (pg/ml)	82 ± 14	96 ± 9	136 ± 21[A,b]
IL-6 (pg/ml)	3 ± 2	10 ± 5	33 ± 4[A,B]
CRP (mg/ml)	40 ± 21	45 ± 31	107 ± 44[a]

Values are mean ± standard deviation of the number of animals indicated in brackets. Statistical analysis was carried out using ANOVA followed by Tukey–Kramer post hoc test ([A] $P < 0.001$ and [a] $P < 0.05$ vs control and [B] $P < 0.001$ and [b] $P < 0.05$ vs 8 h).

Table 4
Global associations among parameters in the experimental conditions

	TNFα	IL-1β	IL-6	CRP
HDL-C	−0.23	−0.81**	−0.82**	−0.81**
LDL-C	0.1	0.76*	0.67*	0.39
Apo A-I	−0.35	−0.85**	−0.73**	−0.78**
Apo A-IV	0.1	0.31	−0.23	0.076
Apo C-III	−0.24	0.1	−0.28	−0.27
CRP	0.43	0.87**	0.56*	—

Pearson correlation coefficients r_s are indicated. **$P < 0.001$; *$P < 0.01$.

correlation coefficients observed are suggesting that IL-1β and IL-6 are important determinants of apo A-I, HDL and C-reactive protein concentrations following turpentine treatment in pig.

2.3. Effect of turpentine administration on hepatic expression of the apo A-I/C-III/A-IV gene cluster

The induction of acute phase caused a decrease in *apo A-I* message 48 h after turpentine injection (Fig. 3).

A different temporal pattern was observed for *apo A-IV* where there was a nadir at 8 h followed by a significant recovery after 48 h (Fig. 3). However, the observed values at this time period were still significantly lower than those found in control animals. A quite opposite pattern was shown by *apo C-III* message (Fig. 3), while this mRNA significantly increased at 8 h, a significant decrease was observed 48 h after toxic administration.

2.4. Effect of interleukins 1 and 6 and TNF-α on expression of the apo A-I/C-III/A-IV gene cluster

Cultured pig hepatocytes were incubated in the presence of different cytokines at a concentration of 1000 U/ml for 24 h and gene cluster messages were monitored (Fig. 4). While IL-1 had no effect, IL-6 and TNF-α induced a significant decrease in *apo A-I* message. Likewise, a significant decrease in *apo A-IV* message was also observed after the addition of the three cytokines. In clear contrast, both interleukins IL-1 and IL-6 induced significant increases in *apo C-III* message, and TNF-α had no effect on the expression of this gene.
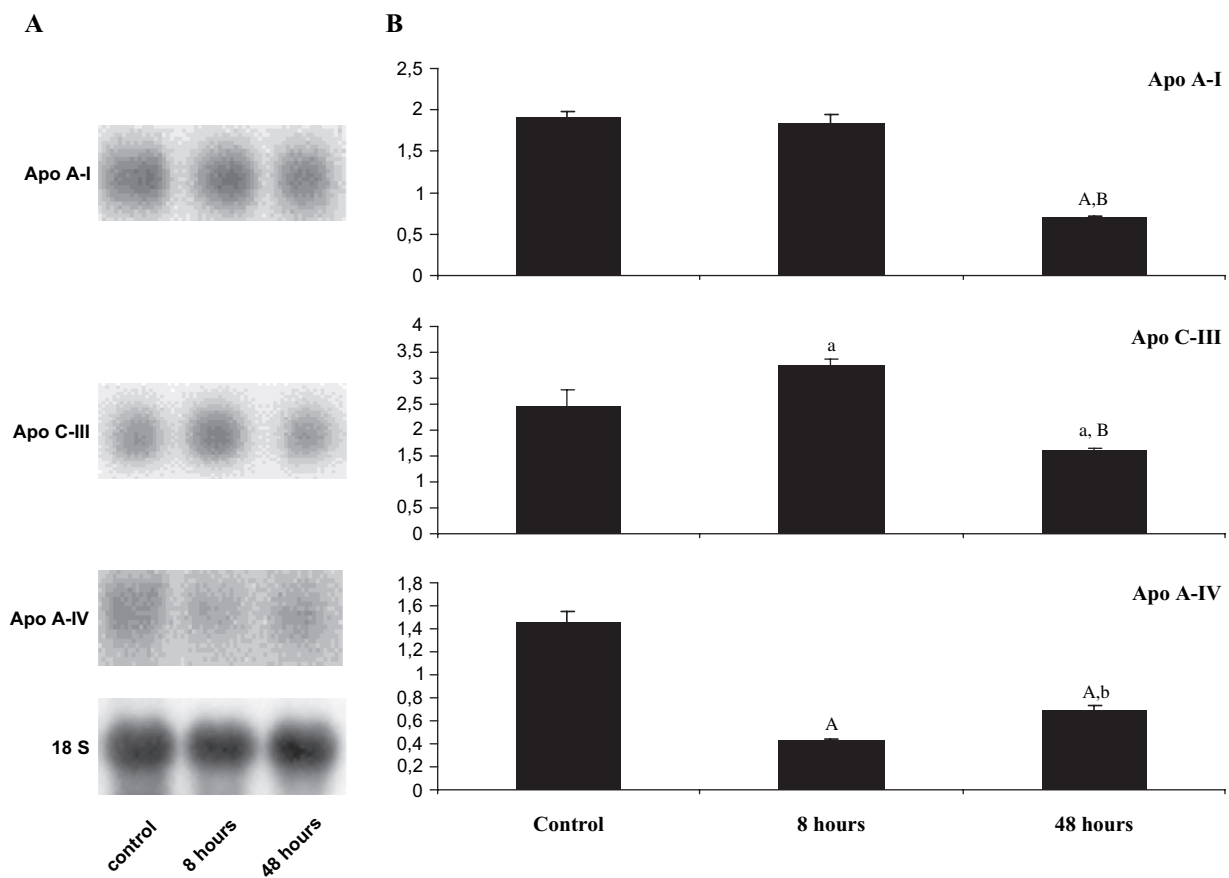

Fig. 3. Effect of inflammation on hepatic apo A-I/C-III/A-IV mRNA gene cluster expression. Inflammation was induced by turpentine oil injection and liver samples were recovered and measured after 8 and 48 h. (A) Representative images of hybridization to different probes. (B) Values are reported as relative arbitrary units (RAU) obtained by Northern-blot analysis of the different messages normalized to the 18 S RNA. Data are mean ± SEM. Statistical analysis was carried out using one-way ANOVA followed by Tukey–Kramer post hoc test. (A, a) $P < 0.001$ and $P < 0.05$ vs control, respectively, and (B, b) $P < 0.001$ and $P < 0.05$ vs 8 h.
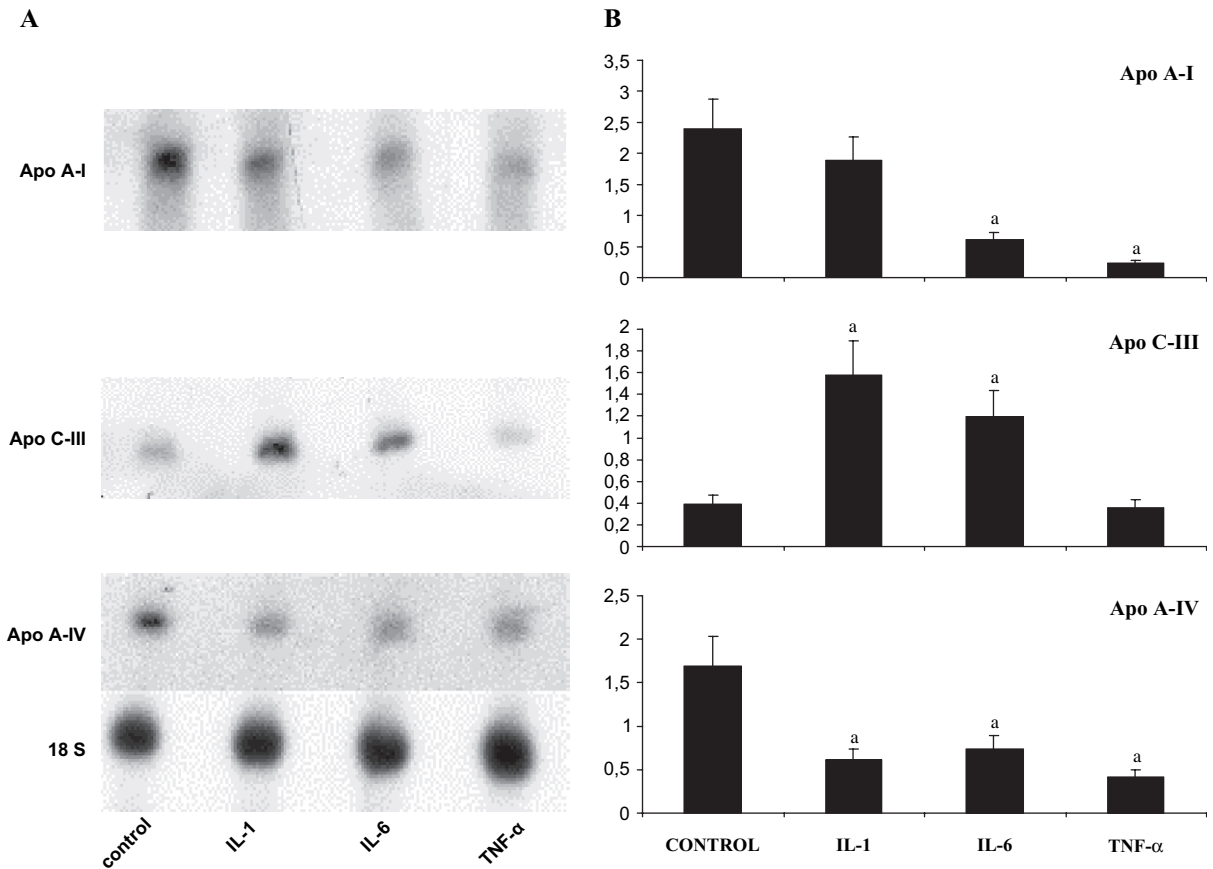
Fig. 4. Effect of cytokines and TNF-α on apo A-I/C-III/A-IV mRNA gene cluster expression in cultured hepatocytes. Porcine isolated hepatocytes incubated in the presence of 1000 U/ml IL-1, IL-6 or TNF-α for 24 h. (A) Representative images. (B) Values are reported as relative arbitrary units (RAU) obtained by Northern-blot analysis of the different messages normalized to the 18 S RNA. Data are mean ± SEM. Statistical analysis was carried out using one-way ANOVA followed by Tukey−Kramer post hoc test. (a) $P < 0.001$ vs control.

3. Discussion

The experimental approach followed has shown that decreased mRNA expression of apolipoproteins A-I and A-IV, HDL constituents, is involved in turpentine acute-phase response in pigs. This expression is also down-regulated by in vitro incubation of hepatocytes with interleukins 6 and TNF-α. A differential regulation exists among the genes belonging to the *A-I/C-III/A-IV* gene cluster in the temporal frame of study as well as in the type of response when analyzed at the steady-state level of mRNA or at plasma levels. The decrease in *apo A-I* mRNA is reflected in the decrease in plasma apo A-I. The latter is the main cause of the decreased HDL levels, and is inversely associated with the levels of circulating IL-6 and IL-1β. However, the decreased levels of *apo A-IV* mRNA are not reflected in its plasma protein, and the decrease in HDL-apo A-IV represents an enlargement of this type of particles. Hepatic *apo C-III* mRNA expression parallels the levels of plasma apo C-III which behaves in agreement with plasma

triglycerides. Overall, inflammation plays an important role in all processes related to the expression of the *A-I/C-III/A-IV* gene cluster.

With the exception of pigs belonging to the hyper-cholesterolemic strain described by Hasler-Rapacz et al. [26], the pig is an animal model with a low level of circulating triglycerides and an extraordinary low concentration of plasma apo C-III ($\sim$50 μg/dl). The pig model has the lowest value when compared to other species (5−15 mg/dl) such as cows [27], humans [28] and mice [29]. Due to this, the earliest reports considered that apo C-III was not present in pig plasma [30]. Afterwards, its presence in plasma was demonstrated using enriched VLDL preparations [31]. However, neither plasma estimation nor biological variations of concentration were ever addressed in this species using common strains. The present report emphasizes the low plasma concentration in pig, despite an important expression at the mRNA level in the liver (main biosynthetic location for this pig apolipoprotein [14]). Furthermore, the present study shows that the protein

apo C-III experiences regulation at the hepatic mRNA as well as the plasma protein levels.

Inflammation studies are gaining interest nowadays due to the fact that inflammatory markers are associated with the prevalence of coronary heart diseases and their control may be an action mechanism of hypolipidemic drugs such as statins [32] and fibrates [33]. Many protocols of inducing acute phase reaction are used: croton oil, lipo-polysaccharide or turpentine injections etc., as well as a great variety of animals such as hamsters, rabbits, baboons, mice and rats [18,20,34]. Previous work to characterize the induced acute inflammation response in pigs showed an increase in concentrations of C-reactive protein, haptoglobin and ITIH4 (formerly pig-MAP) 48 h after turpentine injection [35]. The plasma increased cytokines and CRP levels prove the presence of an inflammatory status although the response was more moderate and delayed than that observed by the administration of lipo-polysaccharide to this animal model [36]. The present work characterizes lipid metabolism after turpentine injection in pigs. Our findings show that plasma cholesterol levels are not modified in these animals. The pig would be an intermediate model between rodents, where their plasma cholesterol increases in APR, and primates where it remains unaltered or even decreases [37]. Furthermore, acute phase induced a transitory increase in plasma (Table 1) and VLDL (Fig. 1B) TG, associated with an increase in hepatic expression (Fig. 2) and plasma apo C-III levels (Table 2). The latter increase could retard the clearance of VLDL [38], a fact that would explain the hypertriglyceridemia found 8 h after turpentine administration. The elevation of apo C-III in plasma adds a new perspective to acute phase reactions, reconciling the discrepant elevations of both plasma TG and lipoprotein lipase activities observed in other animal models considering that apo C-III has been shown to be an inhibitor of the latter enzyme [37]. Thus, the hypertriglyceridemic event has been found to take place at an earlier stage in comparison with other animal models [18]. Likewise, the decreased HDL and the increased LDL (Fig. 1B and C) at 48 h as acute phase markers related to lipid metabolism suggest that they appear earlier in this animal when compared to the time frame required by other animal models to display similar effects [18,34] and justify the absence of change in plasma cholesterol in this animal.

The porcine decrease in HDL and the increase in TG indicate that the *apo A-I/C-III/A-IV* gene cluster is a target for acute phase reaction and requires a special attention for the regulation of the cluster in the inflammatory process in pigs. In acute phase, neither the magnitude of response, nor the behaviour of the three genes was similar. Both results indicate a loss of the coordinated response previously reported for this

animal in other experimental settings [39,40]. The decreased *apo A-IV* mRNA not correlated with levels of plasma apo A-IV reveals that regulation of this gene may be more complex than previously suspected, regarding potential modulators as well as diversity of mechanisms [7].

The decrease in apo A-IV HDL corresponds to an increase in the LDL fraction. This fact suggests a specific enlargement of A-IV-containing particles since no change in apo A-I distribution was observed (Table 2) and an increase in TG was noted in the region corresponding to LDL after 48 h of turpentine administration (Fig. 1). These findings would indicate that the phenomenon of enlargement of HDL with triglycerides in pig acute phase would be limited to HDL particles containing apo A-IV and therefore of very limited magnitude in this species. However, the more pronounced decrease in HDL (Fig. 1C) 48 h after turpentine injection, together with the decrease in plasma apo A-I and the data of *apo A-I* mRNA (Fig. 3) are clearly indicating that acute-phase response has a delayed component where a diminution of production of apo A-I is also involved. The pig is an animal lacking apolipoprotein A-II [41] and with no CETP activity [42]. In this setting, turpentine-induced APR response shows a species-specific response in the sense of decreasing apo A-IV HDL, in contrast to rodents [43], what reinforces the pronounced diminution of this HDL through its major apolipoprotein component.

The acute phase reaction was previously characterized by the porcine hepatocyte system showing an upregulation of ITIH4 and haptoglobin by the action of IL-6 and a down-regulation of albumin [44] by the action of IL-1, TNF-α, and IL-6 in the described conditions. In the present study, addition of the three cytokines to cultured pig hepatocytes caused a general down-regulation of *A-IV* mRNA levels and a selective decrease in apo A-I only by IL-6 and TNF-α. However, *apo C-III* message showed a differential response, while the interleukins IL-1 and IL-6 induced its mRNA expression, TNF-α decreased it. These in vitro observations reproduce the dual behaviour of this gene in vivo. All these data together with the high correlations between apo A-I and interleukin 6 suggest the involvement of this cytokine in the turpentine-induced changes. Likewise, the different response of the gene cluster members further supports the lack of coordination among the members in response to acute phase reaction and an orchestrated intervention of different cytokines at different time periods of injury-healing in vivo.

These effects of interleukins in vitro are also indicating proper maintenance of the cascades transducing gp330 and TNF-α receptor, signals that converge on the regulatory elements of the gene cluster. Thus,

TNF-α, through a complicated signaling cascade, activates AP-1 and NF-kB [45] and IL-6 activates the Janus-associated kinases (JAKs), signal transducer and activator of transcription 3 (STAT3) pathway [46]. The similar effects of TNF-α and IL-6 on *apo A-I* and *apo A-IV* mRNA levels are indicative of a convergent action of both cascades. In this regard, PPAR-α may be a common candidate to be repressed since it either activates or represses both genes depending on the species [47,48]. Treatment with fenofibrate (PPAR-α agonist) suppresses the IL-6-induced acute phase reaction [33]. On the other hand, IL-1 through its receptor activates AP-1, NF-kB and CREB [49]. However, the response of *apo A-I* and *apo A-IV* genes to this cytokine is different, while the former gene is not significantly modified; the second undergoes a dramatic decrease in mRNA levels. The response of *apo A-I* gene to incubation with IL-1 has been variable depending on the cell type. Thus, in HepG2, IL-1 induced significant decreases [50] and in HUH-7, the opposite was found [51]. In this way, pig hepatocytes would represent an intermediate position. It has been proposed that NF-kB mediating action of IL-1 has a different composition as homodimer p50—p50 in contrast when transducing other interleukins [52]. The different composition of NF-kB by itself cannot explain the different behaviour of this interleukin on *apo A-I* and *apo A-IV* genes and thus the intervention of other transcription factors is required. Likewise, the similar action of IL-1 and IL-6 on *apo C-III* expression cannot only be explained by the action of NF-kB. Although the promoter of this gene has a DNA regulatory element recognized by this transcription factor [53], the activation requires the removal of an inhibitor that has not been characterized to date. In this sense, the action of IL-6 repressing PPAR-α action, an inhibitor of *apo C-III* expression [54], would be consistent with the observed induction. On the other hand, TNF-α, an activator of NF-kB, does not induce changes in *apo C-III* message in contrast with the results observed in HepG2 [55]. All these singularities are suggesting a complex interplay of transcription factors modulated by species-specific cascades in response to a specific cytokine, for which further investigation of this hepatocyte system is called for.

In conclusion, the hepatic expression of *apo A-IV* coordinated with the other members of the gene cluster is no longer maintained in a turpentine-induced acute phase. The pleiotropic effects, mediated by interleukins 1 and 6 and TNF-α, may also explain the in vivo induced mRNA changes noted in acute phase, and as a consequence, the plasma changes. The issues raised are interesting aspects for further development of research in this animal model whose apolipoprotein *A-I/C-III/A-IV* gene cluster shows the behaviour described in the present paper.

4. Material and methods

4.1. Animals and study design

Twenty-seven crossbred Landrace and Large-White male pigs (28—39 kg body weight) were used. In 17 animals, an acute inflammation was induced by subcutaneous turpentine injection (0.5 ml/kg). At 8 and 48 h after injection, eight and nine animals, respectively, were employed. As control, 10 animals were injected with equal volume of saline solution. In all groups, after an 8 h fasting period, animals were anesthetized and sacrificed. Plasma and liver samples were obtained. Pieces of tissue were immediately frozen in liquid N_2 until total RNA was extracted. The animals used in this study were handled observing European Union criteria for care and use of laboratory animals in research and the protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

4.2. Plasma parameters

Total plasma cholesterol and TG were quantified using enzymatic methods (Sigma, Madrid, Spain). To analyze plasma lipoprotein profiles, 100 μl of plasma from each animal was subjected to fast protein liquid chromatography gel filtration using a Superose 6B column (Amersham-Pharmacia, Barcelona, Spain), as previously described [56]. 0.5 ml from each fraction was collected. Cholesterol was estimated in fractions with a fluorescent assay using Amplex Red following manufacturer instructions (Molecular Probes, Oregon, USA). TGs were estimated by a new enzymatic procedure with a detection limit of 5 μM. Fifty microlitres of fractions was incubated with 50 μl of reagent solution containing 5000 U/ml of Pseudomonas lipoprotein lipase (Sigma, Madrid, Spain), 1 U/ml of Bacillus glycerol kinase (Sigma, Madrid, Spain), 2.5 mM ATP, 9.5 U/ml Streptomyces glycerol-3-phosphate oxidase (Sigma, Madrid, Spain), 2 U/ml horseradish peroxidase (Molecular Probes, Oregon, USA), 300 μM Amplex Red (Molecular Probes, Oregon, USA) and 1 mg/ml Triton X-100 dissolved in 0.1 M phosphate buffer pH 7.7. Reactions were incubated at 37 °C for 30 min. Intensity of fluorescence was measured in a fluorescence microplate reader (SPECTRAfluor Plus, TECAN) at 595 using an excitation wavelength of 550 nm. The essay was lineal between 5 and 40 μM triglyceride concentrations.

The collected fractions were concentrated by centrifugation in Centricon number 10 tubes, pore size 10 kDa (Amicon Inc. Beverly, MA, USA) and their final volumes measured. Protein content was quantified by Bradford's method [57]. Concentrated samples were analyzed by loading equal amounts of protein on a polyacrylamide gel for Western-blot analysis and thus apo A-IV concentration and distribution in the different

fractions were estimated. Apo A-I was quantified by radial immunodiffusion using a rabbit polyclonal antibody against pig apolipoprotein A-I. The concentration of this apolipoprotein was referred to a standard of apo A-I [58]. An enzyme-linked immunosorbent assay for apo C-III was developed. Serum samples were diluted 5-fold with a 0.2 M sodium carbonate buffer (pH 9.6). Microtitration plates (Maxisorb, NUNC, Roskilde, Denmark) were coated with 100 μl of the diluted samples and incubated overnight at 4 °C. The wells were then blocked with 200 μl of 5% (w/v) non-fat milk in PBS for 1 h at 37 °C and washed four times with 0.2% Tween-20 in PBS. Anti-mouse apo C-III (diluted 1/200, Biodesign, Saco, Maine, USA) (200 μl per well) was added, and the plates were incubated for 90 min at 37 °C. The wells were thereafter washed four times, and 200 μl of goat anti-rabbit IgG serum conjugated to alkaline phosphatase (diluted 5000-fold with 0.2% Tween-20 in PBS) was added to each well. The plates were incubated for 90 min at 37 °C, washed four times, and developed for 20 min with 200 μl of 0.1 (w/v) p-nitro phenylphosphate in 0.5 mM $MgCl_2$, 10% diethylamine buffer pH 9.6. Reaction was stopped with 50 μl of 1 M NaOH. Absorbance was measured at 430 nm, using a microplate reader (SPECTRAfluor Plus, TECAN). All assays were done in triplicate, and all samples were processed in the same day. The intra-assay CV was 1.25%. Concentration of apo C-III in serum is expressed as arbitrary absorbance units/100 μl. Specificity of the anti-mouse antibody was verified by Western-blot analysis.

Plasma levels of interleukins 1β, 6 and TNF-α were determined by ELISA following protocols provided by the suppliers (R&D Systems Inc., Minneapolis, MN, USA and BioSource International, Camarillo, CA, USA). CRP was assayed as previously described [35].

4.3. Western-blot

Plasma samples were loaded onto a gradient (4−24%) SDS-polyacrylamide gel. Electrophoresis was carried out for 3 h at 25 mA and protein transferred to PVDF membranes (Millipore, Madrid) using a transblot transfer apparatus (Biorad) at 400 mA for 18 h. Apolipoprotein A-IV distribution in FPLC fractions was determined by analysis of concentrated fractions obtained from FPLC and subjected to Western-blot analysis. Protein bands were detected using rabbit polyclonal antibodies against human apo A-IV followed by detection using a secondary antibody anti-rabbit IgG peroxidase conjugate (Amersham-Pharmacia) and chemiluminiscence detection (Amersham-Pharmacia). Membranes were exposed to enhanced chemiluminiscent films (ECL, Amersham-Pharmacia) and analyzed using a laser LKB 2202 densitometer (Amersham-Pharmacia).

4.4. Culture of porcine hepatocytes

Liver cells were prepared from pigs aged approximately 12 weeks and weighing between 33 and 43 kg. They had been fed water ad libitum and pelleted food free from additives. Animals were killed by an i.v. injection of 150 mg/kg body weight sodium-pentobarbital, immediately followed by exsanguinations. Hepatocyte isolation was performed as previously described [59]. Liver cells were recovered by centrifugation at $100 \times g$ for 5 min, washed by resuspension in phosphate buffered saline, pH 7.4 (PBS) and centrifugation in the same conditions. The hepatocyte fraction obtained by centrifugation at $50 \times g$ for 2 min, was resuspended in PBS and layered over a 50% Percoll solution (Pharmacia, Uppsala, Sweden) in PBS. After centrifugation at $400 \times g$ 15 min, pelleted cells were washed once with PBS. Isolated hepatocytes were resuspended (10^6 cells/ml) in Williams's medium E containing 5% fetal calf serum, 2 mM glutamine and 50 mg/ml gentamycine-sulphate. Two millilitres of cell suspension was transferred to 35 mm culture dishes and incubated at 37 °C in 5% CO_2 for 12 h to allow cell attachment.

4.5. Cytokine treatment

The culture medium was replaced with fresh medium without fetal calf serum. Recombinant human (rh) cytokines IL-6 (CLB, Amsterdam), IL-1 and TNF-α (ITK diagnostics), at a dose of 1000 U/ml were added to each dish, in the presence of 1 mM dexamethasone that increased the rate of surviving hepatocytes (data not shown). Cells were incubated for 24 h at 37 °C, 5% CO_2. Parallel control experiments were carried out in the absence of the corresponding cytokine [44]. A pool of four experiments (carried out in duplicates) was used for RNA extraction.

4.6. Extraction of total RNA

Total RNA was isolated by the method of Chomczynsky and Sachi [60] using Ultraspec-II (Biotecx Laboratories). One hundred micrograms of tissue was homogenized with 1 ml of the reactive. For hepatocyte cultures, 5×10^6 cells per millilitre of reactive were used. RNA was quantified by absorbance at $A_{260/280}$ (ratio $A_{260/280}$ was greater than 1.75). Integrity of the 28 S and 18 S ribosomal RNAs was verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining.

4.7. Northern-blot analysis

Total RNA (5 μg) was denatured in 18% formaldehyde and 70% formamide at 65 °C for 15 min prior to electrophoresis in a 1% agarose gel containing 2.2 M

formaldehyde. RNA was transferred to a nylon membrane (Hybond-N, Amersham-Pharmacia) by capillary blotting, fixed with 200.000 μJ UV light/cm^2 in an ultraviolet crosslinker (Hoefer, Amersham-Pharmacia) and hybridized at 42 °C for 18 h under standard conditions [61]. A 330 bp pig *apo A-I* probe was generated by PCR using the following oligonucleotides 5′-CAGGAGGAGATGGAGACGT-3′ and 5′-GCG-TTCAGCTTCTTGGAGG-3′. A 171 bp pig *apo C-III* probe was also PCR generated employing the 5′-GGG-GCTGGGTAACCGACAG-3′ and 5′-GGTGGGGAT-GGGAGGGCAT -3′ oligos [14]. Both PCR products were verified by DNA sequencing. A pig *apo A-IV Eco*rI/*Xho*I 0.8 kb fragment was used as a probe [62]. A 0.7 kb *Bam*HI/*Eco*rI rat 18 S probe was used to normalize the amount of RNA loaded on the gel. Probes were labeled using [α-^{32}P]-dCTP and Rediprime (Amersham-Pharmacia). Filters were exposed to Biomax film (Kodak, Amersham-Pharmacia) and films analyzed using a laser LKB 2202 densitometer (Amersham-Pharmacia).

4.8. Statistics

Results of plasma lipids are shown as mean ± SD while data from Northern-blots are expressed as mean ± SEM. An ANOVA test followed by Tukey–Kramer post hoc test was used for multiple comparisons of unpaired observations. Association between variables was assessed by Spearman's rank-order correlation coefficient (r_s) using the statistics package Instat 3.02 for Windows (GraphPad, CA, USA). The differences were considered non-significant when $P > 0.05$.

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