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ACAT-1, Cav-1 and PrP expression in scrapie susceptible and resistant sheep

Research Article

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Abstract: Scrapie is a prion disease for which no means of ante-mortem diagnosis is available. We recently found a relationship between cell susceptibility to scrapie and altered cholesterol homeostasis. In brains and in skin fibroblasts and peripheral blood mononuclear cells from healthy and scrapie-affected sheep carrying a scrapie-susceptible genotype, the levels of cholesterol esters were consistently higher than in tissues and cultures derived from animals with a scrapie-resistant genotype. Here we show that intracellular accumulation of cholesterol esters (CE) in fibroblasts derived from scrapie-susceptible sheep was accompanied by parallel alterations in the expression level of acyl-coenzymeA: cholesterol-acyltransferase (ACAT1) and caveolin-1 (Cav-1) that are involved in the pathways leading to intracellular cholesterol esterification and trafficking. Comparative analysis of cellular prion protein (PrPc) mRNA, showed an higher expression level in cells from animals carrying a susceptible genotype, with or without Scrapie. These data suggest that CE accumulation in peripheral cells, together with the altered expression of some proteins implicated in intracellular cholesterol homeostasis, might serve to identify a distinctive lipid metabolic profile associated with increased susceptibility to develop prion disease following infection.

Keywords: *Scrapie • Prion diseases • Cholesterol homeostasis • Cholesterol esters*

1. Introduction

Prion diseases are fatal neurodegenerative disorders for which no *in vitam* diagnostic tests or effective treatments are currently available. Prion diseases can be inherited, sporadic or transmissible, but in spite of their diverse origins, all forms seem caused by the structural conversion of the cellular prion protein (PrPc) into its pathologic isoform (PrPSc), which is linked to infectivity [1]. PrPc is normally GPI-anchored to specialized cholesterol-rich domains of the plasma membrane, termed lipid rafts or caveolae [2,3]. These membrane domains are detergent-insoluble regions characterized by the presence of free cholesterol (FC), saturated phospholipids and raft-resident proteins [4]. Increasing evidence indicates that subtle intracellular cholesterol changes affect the intracellular processing/trafficking, function and/or activation of raft-resident proteins, including PrPc [2,5-7]. Therefore, a role for cholesterol in the metabolism of PrPc and its conversion into PrPSc has been proposed [7-10].

In normal tissues, approximately 90% of the total cellular cholesterol resides in membrane raft domains as FC, while only a small amount (approximately 1-10%) is found as CE in a cytoplasmic storage form [11]. Because membrane cholesterol appears critical for the function of raft-resident proteins, cells develop

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a highly integrated set of homeostatic mechanisms that finely regulate FC *vs.* CE pools according to the cell's need. In fact, membrane FC is in a dynamic state, moving to the endoplasmic reticulum (ER) in response to changing homeostatic conditions in the cell [12,13]. FC in the ER, if in excess, is converted to CE by ACAT-1, and stored in the cytoplasm as neutral lipid droplets [14]. When cell needs FC for membrane function, or when CE droplets exceed a critical threshold value, CE can be reconverted to FC and recycled to the membrane by cholesterol binding proteins, such as Cav-1 [4,6].

Our recent studies on the role of cholesterol homeostasis in the pathogenesis of Scrapie, revealed abnormal accumulation of CE in the brains and in *ex vivo* peripheral blood mononuclear cells (PBMCs) and skin fibroblast cultures from healthy and scrapie-affected sheep carrying a scrapie-susceptible genotype, when compared to those of sheep with a resistant genotype [15,16]. Similar alterations were observed in mouse neuroblastoma N2a cell lines persistently infected with mouse-adapted 22L and RML strains of scrapie that showed up to 3-fold higher CE levels than parental cells [16]. Moreover, i) scrapie-like prion protein (PrPres)-producing cell populations of subclones from scrapie-infected cell lines were characterized by higher CE levels than clone populations not producing PrPres, and ii) reduction of CE by cholesterol ester metabolism interfering drugs was associated to PrPres inhibition [17]. We thus suggested that abnormal CE levels could identify a phenotype predisposing a cell to the development of pathologic processes involving abnormal activation, and/or processing, and/or trafficking of membrane resident proteins. Other authors, however, found an inhibition of prion infection by using FC lowering drugs (*i.e.* statins) in both *in vitro* [18,19] and *in vivo* [20] prion models.

Thus, in an attempt to better characterize the lipid metabolic state that appears to influence the susceptibility to prion infection, in the present study we compared gene and protein expression of ACAT-1, Cav-1, and PrPc in brains and skin fibroblasts from Sarda sheep carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) prion protein genotype, both without (ARQ/ARQ-) and with (ARQ/ARQ+) Scrapie. In addition, the correlation between expression of ACAT-1 and Cav-1 proteins with that of PrPc was evaluated in skin fibroblast cultures from sheep with the different genotypes.

2. Experimental Procedures

2.1 Brain and skin samples

Female Sarda breed ovines ranging from 2 to 4 years of age, and raised in the same environmental conditions, were used in this study. All samples were collected according to the Principles of Laboratory Animal Care at the experimental farms of the Istituto Zooprofilattico Sperimentale of Sardinia (Italy), and kindly provided by Dr. Ciriaco Ligios. Brain samples were obtained from 3 sheep: two sheep carried the susceptible scrapie genotype ARQ/ARQ, 1 sheep was clinically affected by natural scrapie (ARQ/ARQ+) while the other was healthy and negative for PrP^{Sc} in the brain (ARQ/ARQ-); the third sheep carried the resistant genotype ARR/ARR. All sheep were euthanized with a barbiturate followed by 4 ml of embutramide and mebenzonico-iodide (Hoechst Roussel Vet), brain samples were stored at –80°C immediately after collection and thawed just prior to use. Fresh skin biopsies were obtained from 14 sheep and were delivered to our laboratory in transport medium (Eurocollins). Ten biopsies were from sheep with the susceptible genotype ARQ/ARQ; of these, 2 were scrapie-free (ARQ/ARQ-), while 7 developed clinical disease following experimental inoculation of scrapie and 1 had natural scrapie (ARQ/ARQ+). In addition, skin biopsies from 4 scrapie-resistant ARR/ARR sheep, which were experimentally infected with scrapie and remained free of clinical signs, were used as controls. All biopsies were taken at the terminal clinical stage of the scrapie-affected sheep.

2.2 Skin fibroblast cultures

Tissue fragments from skin biopsies were plated into 6-well plates and allowed to adhere to the bottom of the vessels. After 2 h, they were covered with a few drops of Dulbecco's modified Eagle's medium (D-MEM) (Gibco Lab NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and fungizone (Life Technologies, Inc.) and incubated at 37°C in a humidified incubator with 5% CO_2. The following day, tissue fragments were overlaid with culture medium, which was changed every two days. Five to six days later, fibroblasts begun to proliferate from the fragment margins ("halo of cells") and began to form a monolayer. After four weeks, fibroblasts were purified by repeated trypsinization (trypsin-EDTA) and passaging to achieve an homogenous population of spindle cells. Purified fibroblasts were washed twice with sterile PBS and centrifuged. $1x10^6$ cells were then seeded into 25 cm^2 culture flask and grown to confluence. Then, cells were either used for *"in vitro"* experiments or resuspended in cryopreservation medium at a density of $1x10^7$ cells/ml and stored in liquid nitrogen. Analytical assays were carried out using fibroblast cultures between the second and fourth passage. Cells were plated at a density of $5x10^3$ cell/cm^2 in 6-well plates and brought to proliferative quiescence by incubation for 48 h in serum depleted

(0.2% FCS) MEM 199. Then, quiescent cells were stimulated to re-enter cell cycle by addition of 10% FCS and incubation at 37°C for the indicated time intervals.

2.3 RT-PCR and Southern blotting

The expression levels of Cav-1 and PrP mRNAs were evaluated in brain homogenates and skin fibroblasts by reverse transcription polymerase chain reaction (RT-PCR). mRNA levels for the housekeeping gene β-actin were used to normalize the amount of RNA inputs in the RT-PCR. Total RNA was extracted from 10^6 cells using the reagent TRIZOL (Invitrogen Corporation). Equal amounts of total RNA (1 µg) were reverse transcribed into cDNA using the random hexamer method and amplified by PCR in the presence of specific primers, according to the instructions provided by the manufacturer (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus).

PCR was performed using the following ovine-specific primers and conditions: for Cav-1 (258 bp) forward: 5'- GATTAACAGTGGGTACGATA-3', reverse: 5'-TATGTAGTCTTGCGTTATCC-3'; 94°C for 30 s, 59°C for 30 s and 72°C for 45 s, for 30 cycles. For PrP, (341 bp) forward: 5'-ATTGTCACCTAGCAGATAGA-3', reverse: 5'-TTGTTCAGTAGCTCAAGTCT-3'; 94°C for 30 s, 58°C for 1 min, and 72°C for 45 s, for 30 cycles. For β-actin (217 bp) forward: 5'-GATCATGTTTGAGACCTTC-3', reverse: 5'-GAGGATCTTCATGAGGTAGT-3'; 96°C for 30 s, 60°C for 59 s, and 72°C for 45 s, for 20 cycles. Sub-saturation levels of cDNA templates, needed to produce a dose-dependent amount of PCR product, were defined in initial experiments by testing a range of template concentrations. Amplicons were labeled during PCR with Digoxigenin-11-dUTP (DIG; Roche Applied Science), immuno-detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche Applied Science) and visualized with the chemiluminescent substrate CSPD®. The intensity of the bands was measured after exposure to X-ray film with the Kodak Digital Science Band Scanner Image Analysis System containing HP ScanJet, ID Image Analysis Software. The overall procedure was normalized by expressing the amount of PCR products for each target mRNA relative to the amount of PCR products obtained for the housekeeping gene β-actin.

2.4 Western blotting

Proteins were extracted from brain homogenates and fibroblast monolayers with 0.05 mL per mg of tissue, or cell pellets from 10^6 fibroblasts of RIPA buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1 mM Na_2-EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mM ß-glycerophosphate; 1 mM Na_3VO_4; 1 µg/ml leupeptin). Protein concentration was determined with the Bicinchoninic Acid Protein determination kit (Sigma). Aliquots of protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Millipore, Bedford MA). Blots were subjected to immunoblotting with a 1:5000 dilution of the anti-ACAT-1 antibody (H-125, Santa Cruz Biotechnology, Santa Cruz, CA) and a 1:2000 dilution of the anti-Cav-1 antibody (7C8, Novus Biologicals, Littleton CO), as indicated by the manufacturer. Blots were then reacted with the appropriate dilution of a HRP-conjugated secondary antibody (1:6500 for ACAT-1 and 1: 1000 for Cav-1). Specific bands were detected after addition of the chemiluminescent substrate (Amersham, Freiburg Germany), and analyzed by the NIH Image 1.63 Analysis Software program (Scion Image).

2.5 Statistical analysis

Data are reported as mean ± standard error (SE). Statistical calculations were performed using the statistical analysis software of Origin 7.0 version (Microcal, Inc, Northampton, MA, USA). A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1 ACAT-1 protein levels in sheep brains

Due to the role of ACAT-1 in the conversion of FC into CE, we investigated whether the different CE levels, previously observed in brains of scrapie-resistant and scrapie-susceptible sheep [15], correlated with the expression of this enzyme. Thus, Western blot analysis of brain protein extracts of ARR/ARR (scrapie-infected; scrapie-free), ARQ/ARQ$^-$ (mock infected) and ARQ/ARQ$^+$ (scrapie-infected; scrapie-affected) sheep was carried out using the anti-ACAT-1 H-125 monoclonal antibody. As shown in Figure 1, lower expression levels of ACAT-1 enzyme were indeed detected in the brains of ARR/ARR sheep, with respect to those of ARQ/ARQ$^-$ and ARQ/ARQ$^+$ animals. When compared to sheep with ARR/ARR genotypes, ARQ/ARQ$^-$ and ARQ/ARQ$^+$ sheep showed 1.7 and 2-fold higher levels of ACAT-1, respectively.

3.2 PrPc and Cav-1 mRNA levels in sheep brains

Based on the knowledge that PrPc is a raft-resident protein [2,3], and that Cav-1 [4,6] is involved in FC trafficking, we next measured mRNA levels of PrPc and Cav-1 in brain homogenates of scrapie-susceptible and scrapie-resistant sheep. Unfortunately, ACAT-1 mRNA levels could not be evaluated because the sequence

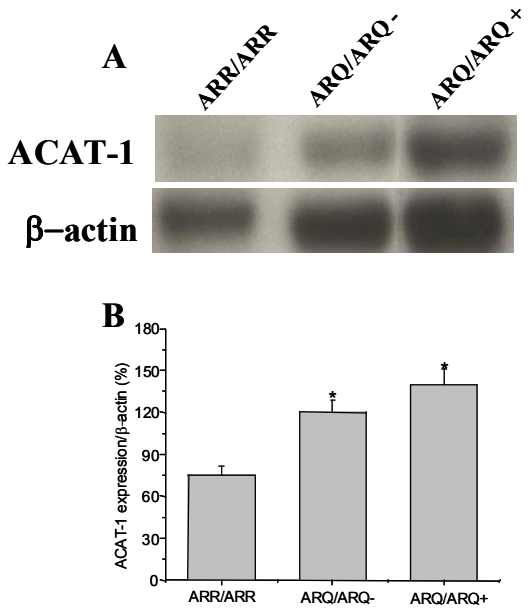

Figure 1. ACAT-1 protein expression in brain extracts of sheep carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) genotypes, with (ARQ/ARQ+) or without (ARQ/ARQ-) clinical Scrapie. Proteins were extracted, separated by PAGE and subjected to Western blot as indicated in Materials and Methods. Panel (A) shows representative chemiluminescent Western blots of ACAT-1 and ß-actin, used as an internal control. Panel (B) shows densitometric analyses of the ACAT-1 bands shown in panel A, normalized for the endogenous ß-actin protein content. Data represent mean values ± SE of triplicate determinations of four samples from each brain. *P<0.05 *vs.* ARR/ARR.

of the ovine ACAT gene is not available. As shown in Figure 2, lower levels of PrPc mRNA, and higher levels of Cav-1 mRNA, were detected in ARR/ARR sheep brains as compared to ARQ/ARQ$^-$ and ARQ/ARQ$^+$ animals.

3.3 ACAT-1 and Cav-1 protein levels in quiescent and replicating sheep skin fibroblasts

Since alterations in cholesterol homeostasis were also found in peripheral cells [15,16], we investigated whether similar expression patterns of ACAT-1 and Cav-1 differentiated skin fibroblasts from sheep with different genetic susceptibility to Scrapie. Due to the fact that cholesterol homeostasis is differently modulated during cell growth [21], the expression level of the two proteins was analysed at various time points after growth stimulation with FCS. Western blot analysis (Figure 3AB) confirmed that, consistent with the results obtained in brains, quiescent (time 0) ARR/ARR fibroblasts contained remarkably lower ACAT-1 expression than ARQ/ARQ fibroblasts. Following FCS stimulation, the above differences in ACAT-1 expression became less prominent, with the exception of ARQ/ARQ$^-$ fibroblasts at 24-48 h. On the contrary, ARR/ARR quiescent fibroblasts showed remarkably higher Cav-1 expression than ARQ/ARQ fibroblasts. Also in this case the above differences became less dramatic 24 h after FCS stimulation. In summary, scrapie-susceptible animals exhibit higher levels of ACAT-1 protein in both fibroblasts and brains. By contrast, significantly lower amounts of Cav-1 protein expression, particularly at 0 time and 24 h after growth stimulation, characterized the scrapie-susceptible sheep fibroblasts.

3.4 Cav-1 and PrPc mRNA levels in quiescent and replicating sheep skin fibroblasts

As shown in Figure 4, with respect to ARR/ARR fibroblasts, ARQ/ARQ cultures associated basal (time 0) had lower mRNA levels of Cav-1 to higher mRNA levels of PrPc. Following FCS stimulation, the differences between scrapie-susceptible and scrapie-resistant sheep in the Cav-1 and PrPc mRNAs diminished or disappeared at 24 and 48 h, to reappear at the 72 h time point.

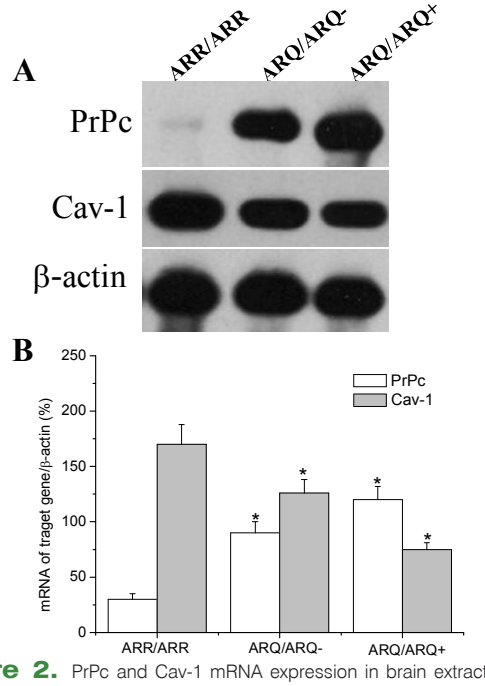

Figure 2. PrPc and Cav-1 mRNA expression in brain extracts of sheep carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) genotypes, with (ARQ/ARQ+) or without (ARQ/ARQ-) clinical Scrapie. Panel (A) shows representative RT-PCR chemiluminescent blots of PrPc and Cav-1 (see Experimental Procedures). Panel B shows densitometric analyses of the PrPc and Cav-1 bands shown in panel A, normalized for the endogenous ß-actin mRNA content that was used as an internal control. Data represent mean values ± SE of triplicate determinations of four samples from each brain.*P<0.05 *vs.* ARR/ARR.

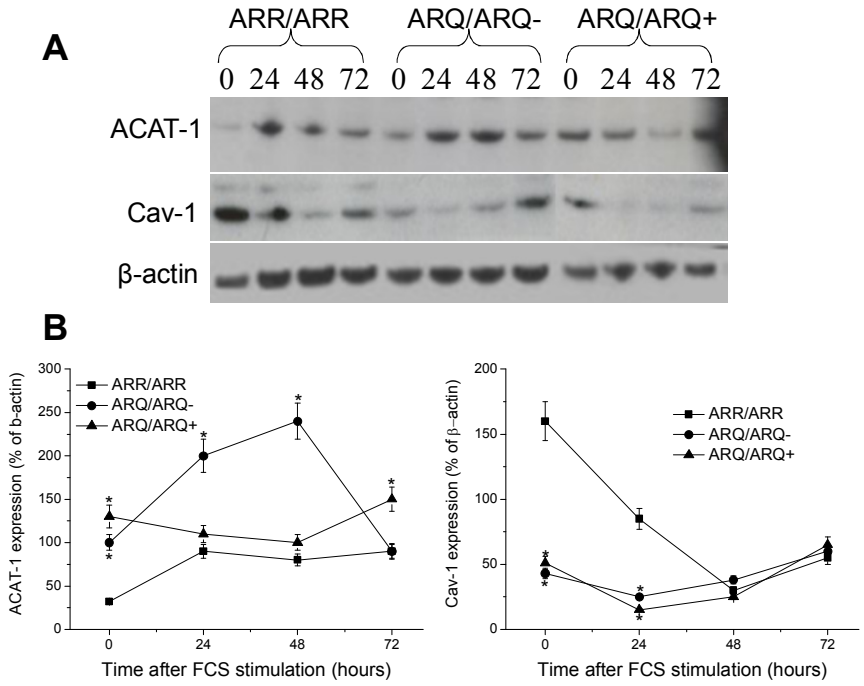

Figure 3. ACAT-1 and Cav-1 protein expression in cultured skin fibroblasts from sheep carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) genotypes, with (ARQ/ARQ+) or without (ARQ/ARQ-) clinical Scrapie. Western blots were performed as described (Experimental Procedures) at the time of growth stimulation with FCS (0 h) or 24, 48 and 72 h later. The upper part (A) shows representative chemiluminescent Western blots of ACAT-1 and Cav-1. The bottom parts (B) show densitometric analysis of ACAT-1 and Cav-1 bands, normalized for the endogenous ß-actin protein content. Bars indicate mean values ± SE of skin fibroblast samples from 4 ARR/ARR, 4 ARQ/ARQ- and 6 ARQ/ARQ+ sheep. *P<0.05 *vs.* ARR/ARR sheep.

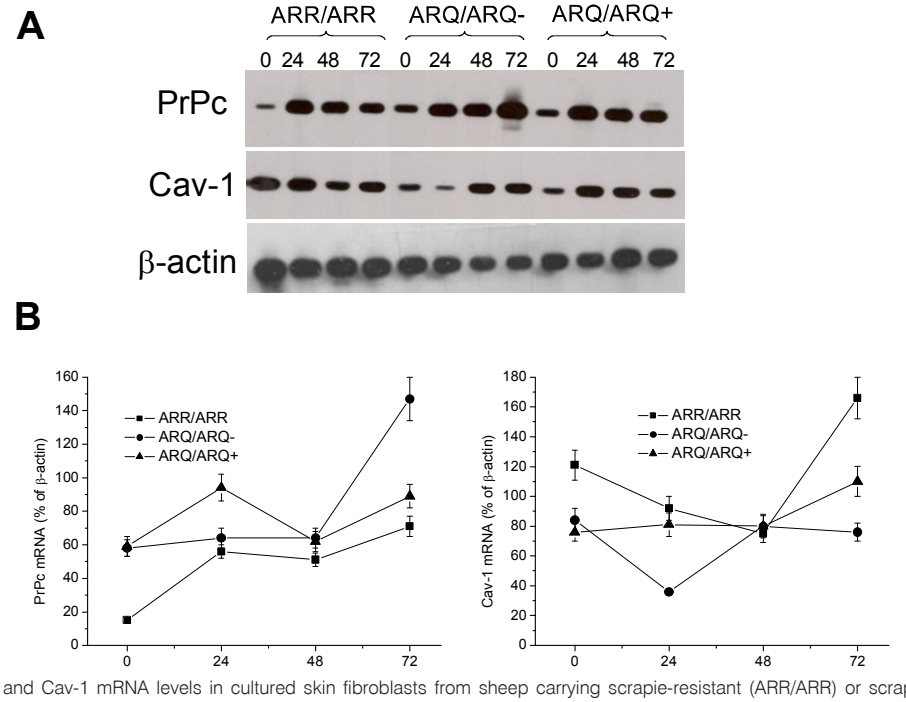

Figure 4. PrPc and Cav-1 mRNA levels in cultured skin fibroblasts from sheep carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) genotypes, with (ARQ/ARQ+) or without (ARQ/ARQ-) clinical Scrapie. Analyses were performed at the time of growth stimulation with FCS (0 h) or 24, 48 and 72 h later. The upper part (A) shows representative chemiluminescent blots of RT-PCR analysis of PrPc and Cav-1, respectively. The bottom part (B) shows densitometric analysis of chemiluminescent bands for PrPc and Cav-1 mRNA, normalized for the ß-actin mRNA contents. Bars indicate mean values ± SE of skin fibroblast samples from 4 ARR/ARR, 2 ARQ/ARQ- and 8 ARQ/ARQ+ sheep. *P<0.05 *vs.* ARR/ARR sheep.

4. Discussion

PrPSc is generally thought to represent the infectious agent of Scrapie, through its ability to promote the structural conversion of the normal cellular prion protein PrPc into a likeness of itself [22]. Although the existence of genetic susceptibility to scrapie infection is well documented [23,24], how particular metabolic states of target cells would modulate degree of susceptibility is not entirely clear. In this context, our previous studies on the role of alterations of intracellular cholesterol homeostasis in the pathogenesis of Scrapie in sheep, revealed a positive correlation between an abnormal accumulation of CE and the genetic susceptibility to infection [15-17]. Of particular relevance, these alterations were found not only in brains but also in peripheral tissues, such as cultured skin fibroblasts and PBMCs, from the scrapie-susceptible sheep (ARQ/ARQ genotype). Moreover, both susceptible and scrapie-affected sheep showed abnormally low levels of high density lipoprotein-cholesterol in their plasma, as compared to resistant animals. Interestingly, Safar *et al*. [25] reported on an interaction of PrPsc with low density lipoprotein suggesting a direct mechanism between cholesterol transport/metabolism and prion biology. In the present study, we found that cellular cholesterol alterations were accompanied by parallel alterations in the expression levels of genes and gene products, ACAT-1 and Cav-1, involved in the pathways leading to intracellular cholesterol esterification and trafficking. In particular, higher ACAT-1 and lower Cav-1 expression characterized cells with scrapie-susceptible genotypes. Interestingly, in fibroblasts carrying the susceptible ARQ/ARQ genotype, we also found that mRNA expression of PrPc was higher than in cultures from scrapie-resistant sheep. These data suggest that genetic PrP polymorphisms and alterations of cholesterol homeostasis may act in concert to create an environment favorable to the initiation and progression of prion disease. Our conclusions are sustained by recent evidence indicating that the metabolic fate of PrPc is dependent on its location at the plasma membrane [26,27], which in turn is mediated by cholesterol levels in the raft domains [28], suggesting that PrP misfolding might be, at least partly, dependent on cholesterol homeostatic regulatory mechanisms. At present, however, although there are clear indications that cholesterol-enriched microdomains (rafts) are essential for the proper folding of nascent PrPc protein, there are divergent findings regarding whether cellular cholesterol depletion or its enrichment would favour the misfolding of PrP. A number of studies have, in fact, shown a decrease of PrPsc generation by lowering the cellular cholesterol content with statins [7,19,20], while other studies produced evidence that cholesterol depletion abolishes PrP-raft association, promotes its accumulation and increases substantially its misfolding [28,29]. In addition, replication of misfolded PrP protein was reported to interfere with the raft composition by displacing raft proteins, such as Cav-1, likely leading to alterations of intracellular cholesterol trafficking and accumulation [30].

In summary, further studies are necessary to elucidate the mechanisms responsible for the cholesterol homeostasis alterations in sheep carrying a genotype susceptible to scrapie, and to unveil possible cause-effect relationships between PrP polymorphisms and cholesterol alterations. Similarly, additional work is needed to reveal the potential implications of this association for prion disease susceptibility. Nonetheless, the data reported in the present paper suggest that accumulation of CE in peripheral cells, together with the altered expression of some proteins implicated in intracellular cholesterol homeostasis, might serve to identify a distinctive lipid metabolic profile (PCT/IT2007/000109; PCT/IT2007/000110) associated with increased susceptibility to develop prion disease following infection.

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