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Adiponectin affects lung epithelial A549 cell viability counteracting TNFα and IL-1β toxicity through AdipoR1

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ABSTRACT

Adiponectin (Acrp30) exerts protective functions on metabolic and cellular processes as energy metabolism, cell proliferation and differentiation by two widely expressed receptors, AdipoR1 and AdipoR2. To date, the biological role of Acrp30 in lung has not been completely assessed but altered levels of Acrp30 and modulated expression of both AdipoRs have been related to establishment and progression of chronic obstructive pulmonary disease (COPD) and lung cancer. Here, we investigated the effects of Acrp30 on A549, a human alveolar epithelial cell line, showing how, in a time and dose-dependent manner, it decreases cell viability and increases apoptosis through ERK1/2 and AKT. Furthermore, we examined the effects of Acrp30 on A549 cells exposed to TNFα and/or IL-1β, two potent lung inflammatory cytokines. We showed that Acrp30, in dose- and time-dependent manner, reduces cytotoxic effects of TNFα and/or IL-1β improving cell viability and decreasing apoptosis. In addition, Acrp30 inhibits NF-κB nuclear trans-activation and induces the expression of the anti-inflammatory IL-10 cytokine without modifying that of pro-inflammatory IL-6, IL-8, and MCP-1 molecules *via* ERK1/2 and AKT. Finally, specifically silencing AdipoR1 or AdipoR2, we observed that NF-κB inhibition is mainly mediated by AdipoR1. Taken together, our data provides novel evidence for a direct effect of Acrp30 on the proliferation and inflammation status of A549 cells strongly supporting the hypothesis for a protective role of Acrp30 in lung. Further studies are needed to fully elucidate the Acrp30 lung effects *in vivo* but our results confirm this adipokine as a promising therapeutic target in lung diseases.

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1. Introduction

Adiponectin (Acrp30) is an adipokine with beneficial effects on a number of biological and metabolic processes including energy metabolism, cell differentiation and proliferation. Acrp30 is abundantly secreted by adipocytes and circulates at high levels (~0.01% of the total protein) (Brochu-Gaudreau et al., 2010). The human gene encoding Acrp30 (ACDC) is located on chromosome 3q27 where single-nucleotide polymorphisms have been associated with obesity and related diseases (Daniele et al., 2008; Gable et al., 2007).

Acrp30 acts through binding and activation of AdipoR1 and AdipoR2, two receptors widely expressed in several organs, tissues and cell lines (Kadowaki and Yamauchi, 2005; Lang and Ratke, 2009; Buechler et al., 2010). AdipoR1, AdipoR2 exert similar effects but the former has been more related to metabolic functions, whereas the latter has been mainly involved in anti-inflammatory and anti-oxidative mechanisms (Yamauchi et al., 2007). How the Acrp30 signals are transduced remains largely unknown but downstream of AdipoRs, the biological effects of Acrp30 are mainly mediated by signal pathways involving ERK1/2, AKT, P38 and AMPK (Brochu-Gaudreau et al., 2010). The beneficial effects of Acrp30 in metabolic diseases (Daniele et al., 2011; Gu and Li, 2012) as well as in several malignancies have been well documented (Vona-Davis and Rose, 2007; Lang and Ratke, 2009). However, in inflammation and

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autoimmune disorders, the exact role of Acrp30 and the underlying molecular mechanisms have not yet been fully clarified (Summer et al., 2009; Ouchi and Walsh, 2008; Miller et al., 2009; Krommidas et al., 2010; Robinson et al., 2011).

Lung cancer and chronic obstructive pulmonary disease (COPD) are among the major causes of morbidity and death worldwide (Rabinovich and MacNee, 2011).

There is a growing interest on alterations in multiple metabolic pathways in both COPD and cancer (Daniele et al., 2012; Atzori et al., 2011). Identification of specific metabolic pathways is required to better understand the intimate mechanisms by which metabolic processes interact with Inflammation and cell proliferation. Although acting through separate pathways, lung inflammation is a crucial component of both neoplastic and non-neoplastic lung disorders including COPD and NSCLC.

A critical site for the complex interplay between environmental triggers, airway inflammation (Mazzarella et al., 2007, 2012; Esposito et al., 2012) and specific metabolic pathways involvement is airway epithelium.

Inflammation triggers lung epithelium and macrophages to release multiple pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) that, in turn, propagate and prolong the inflammation (Murugan and Peck, 2009). A pivotal role in the development of acute and chronic inflammatory diseases is played by NF-κB, a transcriptional factor involved in critical cellular processes such as inflammation, injury and stress (Tang et al., 2006; Imanifooladi et al., 2010). Among lung inflammation mediators, Acrp30 has attracted growing interest since its altered levels as well as the modulation of both AdipoRs in normal, COPD and neoplastic lesions of lung have been described (Petridou et al., 2007; Miller et al., 2009; Daniele et al., 2012). These observations strongly suggest a direct function of Acrp30 on lung although its role is far from being completely defined; in fact, both pro-inflammatory and anti-inflammatory properties have been reported (Summer et al., 2009; Ouchi and Walsh, 2008; Miller et al., 2009; Krommidas et al., 2010).

In the light of this evidence, we designed an experimental study to assess the human alveolar epithelial cell line (A549) response to Acrp30 treatment, in normal as well as in inflammatory conditions. We first evaluated the expression of AdipoRs in A549 cells and successively investigated the effects of Acrp30 on the A549 cell viability, apoptosis and the activation status of the main downstream molecular targets. Finally, we studied the Acrp30 effects on A549 cells following stimulation by TNFα and IL-1β, two relevant cytokines in inflammatory mechanisms of airway disease.

2. Materials and methods

2.1. Cell culture and transfection

Cell lines derived from lung, A549 (DSMZ), SW-1573 (ATCC), Calu-3 (ATCC), were provided from the Bank of Human and Animal Continuous Cell Lines CEINGE-Biotecnologie Avanzate. shRNA constructs against AdipoR1-R2 were obtained from OriGene-Technologies (Rockville, MD). Transfection of A549 cells was performed using LipofectAMINE™ 2000 (Invitrogen, CA) according to the manufacturer's instructions.

2.1.1. MTT test

A549 cells were incubated after 12 h starvation with purified human recombinant Acrp30 (5 or 50 μg/ml) (Biovendor, Germany) and/or TNF((50 ng/ml) and/or IL-1((10 ng/ml) in 5%FBS medium (Sigma–Aldrich, MO). As control, cells were incubated with medium. After 24, 48, 72 h of incubation, cells were stained with MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide/PBS). The experiments were performed two times in triplicate.

2.1.2. Western blotting

A549 cells were treated, after 12 h starvation, in 5% FBS medium according to scheme in Table 1. As control, cells were incubated with medium. After incubation period, proteins from cells as well as from human lung, porcine skeletal muscle and liver tissues were separated on SDS-PAGE as previously described (Daniele et al., 2008). The membranes were incubated with AdipoR1, AdipoR2, GAPDH (SantaCruz-biotechnology, CA), p-ERK1/2, ERK1/2, p-AKT, AKT, p-P38, P38, p-AMPKα, AMPKα and Caspase-3 (Cell-Signaling Technology, MA) antibodies according to the manufacturer's instructions.

2.1.3. Confocal microscopy

A549 cells were seeded in 6-well chambers (Ibidi GmbH), starved for 12 h and incubated in 5%FBS medium with TNFα (20 ng/ml), IL-1β (10 ng/ml), Acrp30 (5 or 50 μg/ml) according to the following scheme: (1) TNFα or IL-1β (20 min); (2) Acrp30 (14 h) washing and incubation with TNFα or IL-1β (20 min). As control, cells were incubated with medium. A549 cells, transiently transfected with ShRNA for AdipoR1 or AdipoR2, were treated as above. After incubation, cells were fixed with 3.7% paraformaldehyde, permeabilized by PBS/TritonX100 0.1% 10 min RT. Immunofluorescence assays were performed using AdipoR1, AdipoR2 (Phoenix Pharmaceuticals, CA), NF-κB primary antibodies and Alexa488-546 conjugated secondary antibodies (BD-Biosciences, CA); DAPI stained nuclei. The samples were analyzed with a LSM510 meta-confocal microscope (Carl Zeiss, Jena).

2.1.4. RNA extraction and real-time PCR

A549 cells, after 12 h starvation, were treated in 5%FBS according to scheme in Table 1. As control, cells were incubated with medium. After incubation, total RNA was isolated using TRIzol (Invitrogen, CA) and Real-time PCR was performed as previously described by Krommidas et al. (2010). The primers for IL-10, IL-6 IL-8, MCP-1 are available on request. The experiments were performed two times in triplicate.

2.1.5. Statistical analysis

Data are means ± SE. Two groups were compared with 2-tailed unpaired Student's *t*-test. Multiple comparisons were performed by ANOVA test. All statistical analyses were performed using the StatView software 5.0.1.0. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. AdipoRs are expressed in A549 cells

We demonstrated AdipoR1 and R2 expression in A549 cells at both mRNA (Supplementary Fig. 1A and B) as well as at protein level (Supplementary Fig. 1C). We validated our results on human and murine normal lung tissues, on skeletal muscle and liver porcine tissues used as positive controls (Supplementary Fig. 1A–C). Immunofluorescence experiments showed the presence of both receptors on plasma-membrane of A549 cells (Supplementary Fig. 1D). Moreover, we demonstrated both AdipoR1 and R2 at mRNA and protein level in two other human lung cell lines SW-1573 and Calu-3 (data not shown).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.03.003.

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