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Title: T cell based RAS activity and insulin levels in obese subjects with low grade inflammation

Short Title T cell renin angiotensin system in obese subjects with insulin resistance

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ABSTRACT

Background

Obesity is a major contributor to inflammation and oxidative stress that are key underlying causes for insulin resistance (IR) and diabetes.

Accumulated evidence suggest that RAS may serve as a strong link between IR and obesity. We investigated RAS activity in circulating T-cell by obese subjects with and without angiotensin (Ang) II stimulation in presence or not of IR and of low grade inflammation.

Materials and methods

We studied 29 obese and 10 healthy subjects. After T-lymphocytes isolation, mRNAs for angiotensin converting enzyme (ACE) and angiotensin (AT)₁-receptor (R) were quantified by RT-PCR. HsCRP, insulin and inflammatory cytokines serum levels, plasma renin (PR) and ACE activity in cell pellet and supernatant, and angiotensin (Ang) II T-cell content were also measured.

Results

Under baseline conditions RAS gene expressions, ACE activity and Ang II levels in T-cells, but not PRA, by obese subjects with or without IR and with or without hs-CRP ≥ 3 mg/dl were ($p < 0.05$) higher than in controls. The increase in all parameters induced by Ang II was significantly higher in T-cells from the obese subjects with hs-CRP ≥ 3 mg/dl than in controls or in the obesities with hs-CRP < 3 mg/dl. In the obese subjects with low grade inflammation and insulin resistance (IR), the cytokine serum levels and T cells RAS gene expression resulted inversely correlated with insulin serum concentration.

Conclusions

Low grade inflammation amplifies the T-cell RAS response to Ang II stimulation. T-cell RAS gene expressions and serum levels of inflammatory cytokines were inversely related with insulin serum

concentration. A protective role of insulin towards the development of inflammatory events can be hypothesized.

Keywords: insulin resistance, Angiotensin II, T-cell, obesity, renin-angiotensin system

INTRODUCTION

The renin angiotensin system (RAS) contributes to the underlying pathophysiology of insulin resistance (IR); however, the optimum approaches to target the RAS for personalized prevention and treatment of IR are still under debate.

The effects of the systemic RAS on blood pressure regulation and glucose metabolism have been studied extensively; recent discoveries on the influence of local tissue RAS in the skeletal muscle, heart, vasculature, adipocytes, and pancreas have led to an improved understanding of how activated tissue RAS influences the development of IR and diabetes in humans.

Namely, IR and type 2 diabetes were prevented by the blockade of RAS in some, but not all patients, suggesting that inter-individual differences contribute to the effects of the RAS on the development of IR (1,2)

Angiotensin (Ang) II is the predominant RAS component contributing to IR. In fact, Ang II is one of the major pro-inflammatory adipokines produced by obese adipose tissue that may be critical in linking obesity, inflammation and IR (3-5).

In the initial stage of inflammation, in addition to pro-inflammatory events, the anti-inflammatory molecules can have an important role: in particular, insulin exerts anti-inflammatory effects by suppressing several factors of innate and adaptive immune response (6-8). Several data reported in the literature show that insulin exerts anti-inflammatory effects by suppressing plasma concentrations and expression of the chemokines and their receptors (CCR)-2 and CCR-5 in mononuclear cells (9). The inflammatory status was clearly reported to be an important factor in diet-induced IR, but it remains to be demonstrated what are the initiation steps involved in

generating the inflammatory cascade. Obese subjects without clinical and biochemical alterations could represent the initial stage of inflammation in obesity.

In this study we investigate RAS activity in cultured circulating T-cells by obese subjects with and without Ang II stimulation in presence or not of IR and in presence or not of low grade inflammation.

METHODS

Subjects

T-lymphocytes were obtained from 29 subjects 43-67 years old, all with BMI >30 who attended to our out-patients' clinic. Ten healthy subjects comparable for age and sex, were studied as the control group.

No patient was on treatment with ACE inhibitors, AT1 R antagonist or diuretics. Exclusion criteria were both primary and secondary forms of arterial hypertension, diabetes mellitus or other metabolic abnormalities, ischemic heart disease/acute coronary syndrome or stroke in the previous 6 months, left-ventricular (LV) ejection fraction <50%, renal failure (creatinine clearance <60 ml/min), presence of a major illness such as cancer, liver disease, chronic and acute inflammatory disease or infectious disease in the previous 3 months. All these exclusion criteria can induce an activation of systemic and T-cell RAS. No subject was in treatment with anti-inflammatory drugs.

Main demographic and clinical characteristics of enrolled patients are shown in Table 1. According to the high sensitivity-C reactive protein (hs-CRP) values at enrolment, subjects with obesity were divided in two groups according to hsCRP value ≥ 3 mg/l or < 3 mg/l. IR was calculated using Homeostatic Model Assessment (HOMA) from fasting plasma glucose (in mmol/L) multiplied by plasma insulin (in IU/L) and divided by 22.5 (10). Individuals with HOMA-IR >2.5 were considered to have IR.

Blood hsCRP level and other general chemical tests are analyzed as a part of a routine medical check-up. The protocol of this study complies with the principles of the Helsinki declaration. The study was approved by the internal review board and written, informed consent was obtained from each subject.

Experimental procedures

T-lymphocyte isolation

T lymphocytes isolation was performed as already reported (11). The stimulation of T cells was done using 0.05 nmol/L of Ang II because this concentration is close to human Ang II plasma values (12).

Real-Time PC for ACE gene expression and Reverse Transcriptase-Polymerase Chain Reaction analysis for AT1R gene expression

Isolation of total RNA and RAS gene expression analysis were performed as previously described (11).

Measurement of serum adiponectin, IL6 and TNF alpha

Human total adiponectin, interleuchin (IL) 6 and tumor necrosis factor (TNF) alpha measurements were performed in serum by commercial kits (Adiponectin ELISA human art 10-1193-01, Mercodia, Sweden; ELISA kit for IL6 art. SEA079Hu DBA Italia Srl; ELISA kit for TNF alpha art SEA133Hu DBA Italia Srl).

Measurement of Ang II concentration in T-cells

Ang II concentration in T cell pellet was performed as previously described (13) Ang II levels were expressed as fmol/mg of proteins.

Measurement of ACE activity.

ACE activity measurement was performed as previously described (13)

Measurement of PRA

PRA measurement was performed with a commercial kit (Sorin Biomedica, Italia)

hsCRP assay

hsCRP was measured by a commercial kit (Beckman, CA, USA) with the lowest detection point of 0.1 mg/l.

RESULTS

PRA did not differ between obese subjects and controls.

ACE and AT1R gene expression in basal conditions in control and obese subjects with or without low grade inflammation and in presence or not of IR.

In control subjects, ACE and AT1R-mRNA expression is present on T-lymphocytes, and did not modify during the 24 hour of incubation without Ang II addition to the culture medium (Fig 1).

Under baseline conditions all the subjects with obesity, in presence or not of low grade inflammation, showed significantly higher levels of ACE and AT1R gene expression ($P<0.001$) in comparison to the control subjects (Fig 1). The presence of IR did not modify the ACE and AT1R gene expression in all the studied groups.

ACE and AT1R gene expression in control and obese subjects with or without low grade inflammation in presence or not of IR after Ang II stimulation.

In the control group the ACE mRNA expression after Ang II addition to culture medium was significantly higher at 18 and 24 hours of incubation respect on under baseline conditions without stimulus (Fig 1). Similarly, T-cell AT1R gene expression significantly increased ($P<0.05$) at the 24th hour when T-lymphocytes were stimulated by Ang II (Fig 1).

In the subjects with obesity without low grade inflammation, we observed a significant early increment of ACE mRNA levels at the 6th and 18th hour of incubation after Ang II stimulation of cultured T lymphocytes. The increase was significantly higher than that observed under baseline condition without stimulus ($P<0.005$). On the contrary in the obese subjects with low grade inflammation the significant increase ($P<0.005$) was observed later, at the 18th hour with a further increment at the 24th hour of incubation; the upregulation of ACE-gene expression was significantly higher in the group of obese subjects with low-grade inflammation than in the obese

subjects with CRP <3 mg/dl (Fig 1). The presence of IR caused only in the subjects with obesity with low grade inflammation a reduced increment of ACE gene expression in response to Ang II stimulation in comparison to the subjects without IR. An inverse correlation was observed between the ACE gene expression at the 18th hour after Ang II stimulation and the levels of insulin in the group of obese with hsCRP > 3 and IR (Fig 3).

In both groups of subjects with obesity under Ang II-stimulation, T-cell AT1R mRNA levels significantly increased at the 6th hour respect on under baseline condition without stimulus ($P < 0.05$). T-cell AT1R mRNA levels further increased at the 18th and 24th hour with a peak level at 18th hour ($P < 0.001$) (Figure 1). During the whole time course AT1R mRNA levels were significantly higher than those observed in controls. In the obese subjects with low grade inflammation and IR a reduced increment of AT1R gene expression in response to Ang II stimulation was observed when compared to the obese subjects with >3 CRP but without IR.

ACE activity in control and obese subjects under baseline conditions and after Ang II stimulation. In control subjects T-cell pellet ACE activity was scarcely detectable at baseline without significant changes during the 24 hours of incubation

In basal conditions ACE activity in T cell pellet and in the supernatant was significantly higher in obese patients in comparison with controls subjects, without differences between obese groups with CRP<3mg/dl and CRP>3mg/dl and in presence or not of IR. Ang II stimulation determined in all obese subjects a significant increase in ACE activity that was released almost completely in the supernatant, mostly in presence of low grade inflammation; only in the patients with usCRP>3 mg/dl, the presence of IR determined a significant ($p < 0.005$) decrease in ACE activity in comparison with obese patients without IR (table 2).

Cell pellet Ang II concentration in control and obese subjects under baseline conditions and after Ang II stimulation.

Control subjects showed a T cell pellet Ang II concentration significantly lower than obese subjects during the whole time course with and without Ang II stimulation.

The obese subjects with hsCRP >3 mg/dl showed a higher Ang II pellet concentration than the obese subjects with hs CRP <3 mg/dl, without significant differences between the two obese groups both at baseline and 24th hour of incubation without Ang II stimulation. The addition of Ang II to culture medium after 24 hours of incubation determined a significant increment ($p < 0.05$) of Ang II pellet concentration in the obese subjects with hsCRP >3 mg/dl in comparison with the obese subjects with hsCRP <3 (Table 2). In the group of obese subjects with hsCRP >3 mg/dl, the presence of IR was associated with a significant decrement of pellet Ang II concentration ($p < 0.005$) respect on the obese subjects without IR.

Adiponectin, IL6 and TNF alpha serum concentration

Baseline serum adiponectin levels were significantly ($p < 0.001$) higher in control subjects than in subjects with obesity in presence or not of low grade inflammation or IR (Fig 2)

Baseline IL 6 and TNF alpha serum concentration were significantly higher ($p > 0.001$) in all subjects with obesity in comparison with control subjects. In the group of obese patients with low grade inflammation and IR the inflammatory cytokines serum concentration were significantly lower ($p > 0.005$) than those of the obese subjects without IR (Fig 2). An inverse relationship was observed between IL 6 and insulin levels in the group of obese with hsCRP > 3 (Fig 4).

DISCUSSION

The obese subjects with low grade inflammation and IR showed a significant ($p < 0.05$) decrease of Ang II-stimulated T-cell ACE and AT1R gene expression when compared with the obese subjects with $\text{hsCRP} \geq 3$ mg/L but without IR. The obese subjects with $\text{hs-CPR} \geq 3$ mg/L and IR also showed a reduction of ACE activity in supernatant and of the concentration of Ang II in T-cell pellet; likewise TNF-alpha and IL-6 serum levels were lower than the levels measured in the obese subjects with $\text{hsCRP} \geq 3$ mg/L without IR. As a whole the presence of IR was associated with a significant decrease of pro inflammatory molecules and T-cell RAS response to Ang II stimulation. An inverse relationship was observed between hsCRP and insulin levels in the obese group with low grade inflammation and IR, but not in the obese group with low grade inflammation but without IR (Fig 5).

IR is a common complication associated with obesity and is secondary to inflammation caused by the infiltration of adipose tissue by monocytes and other circulating mononuclear cells. These cells secrete proinflammatory substances as adipocytokines, adipokines and Ang II, which spill into the bloodstream and contribute to systemic inflammation.

In turn chronic hyperinsulinemia induces the expression of various pro-inflammatory cytokines and oxidative stress (14 -16). In addition, chronic hyperinsulinemia also stimulates the expression of lipogenic genes, fat mass and body weight gain through the regulation of circulating adiponectin level (15) Conversely, our obese subjects with low grade inflammation , $\text{HOMA-IR} > 2.5$ and normal clinical parameters, did not show the expected consequences of IR on some biohumoral parameters involved in the onset of inflammation, as IL-6 and TNF alfa (17); moreover in our obese subjects the hsCRP values are inversely correlated with serum insulin concentrations, suggesting a protective role of insulin against inflammation. In the literature several data reported that insulin can exert anti-inflammatory effects (17). A decrease in proinflammatory plasma

cytokines, intranuclear nuclear factor κ B and Egr-1 binding plasma concentration of MCP1 and RANTES and their receptors CCR2 and CCR5 was reported in mononuclear cells (1). In fact some obese subjects escape the association between obesity and cardio metabolic morbidity and they can represent an early stage of the inflammation.

Under baseline conditions ACE and AT1R mRNA expressions in T-cells from obese subjects were ($p < 0.05$) higher than those observed in T-cells from controls, without differences between T-cells from obese subjects with $> \text{or } \leq 3 \text{ mg/dl}$ hs-CRP and with or without IR. Conversely, the increase in ACE and AT1R mRNAs induced by Ang II stimulation was significantly higher in T-cells from the obese subjects with $\text{hsCRP} > 3 \text{ mg/dl}$ than in T-cells from controls or from the obese subjects with $\text{hsCRP} < 3 \text{ mg/dl}$. The increase in ACE mRNA expression induced by exogenous Ang II strongly supports an increase in Ang II synthesis by cells. On the other hand the contemporaneous augment in the AT1R gene expression do not allow to rule out an intracellular uptake of exogenous Ang II.

In the group of the obese subjects with low grade inflammation, those with IR showed a significant decrease of Ang II-stimulated T-cell ACE and AT1R gene expression and of TNF- α and IL-6 serum levels when compared with the obese subjects with $\text{hsCRP} > 3$ but without IR ($p < 0.05$). So, the presence of IR was associated with a significant decrement in T cells RAS activation and in serum pro-inflammatory cytokines concentration only in the group of obesities with $\text{hsCRP} > 3$; more exactly T-cell ACE and AT1R gene expressions and serum levels of inflammatory cytokines were inversely related with insulin serum concentration. Moreover, in this group the presence of IR was associated with a significant decrement in supernatant ACE activity and in Ang II cell pellet concentration. More exactly, supernatant ACE activity and Ang II pellet concentration measured under baseline conditions are significantly higher in the obese subjects with low grade inflammation respect on controls and the obese subjects without low grade inflammation. The presence of IR reduces ACE activity and Ang II pellet concentration after Ang II stimulation.

Furthermore, in this group, the levels of hsCRP were inversely correlated with insulin concentration and this inverse relationship suggests a protective role of insulin towards the development of inflammatory events. These data can represent the initial stage of inflammatory status in obesity (17).

As already reported in our previous study (18) PRA of control and obese subjects did not significantly differ among the groups studied. Consequently, RAS seems not to have a role in insulin sensitivity at this stage. Experimental studies have shown that in diabetic patients the use of a AT1R blocker, improved insulin sensitivity (17). These data may represent different functional effects of RAS on IR in obese subjects during the different phases of the inflammatory reaction.

In conclusion circulating T-cell based RAS is activated in obese subjects and low grade inflammation amplifies the T-cell RAS response to Ang II stimulation. In the obese subjects with low grade inflammation, T-cell ACE and AT1R gene expressions and serum levels of inflammatory cytokines were inversely related with insulin serum concentration. A protective role of insulin towards the development of inflammatory events can be hypothesized.

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Declaration of Conflicting Interests: The authors have no conflict of interest to declare.

Author Contributions

M.Coppo and M.Boddi conceived the work, L.Poggesi and PA Modesti enrolled the subjects and performed the clinical evaluation, M.Coppo and M.Bandinelli performed the dosages, M.Chiostri performed the statistical evaluation, M.Coppo and M.Boddi wrote the manuscript.

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Figure Legend

Figure 1. ACE and AT1-R gene expression in control and obese subjects.

*p <0.001 vs controls, § p<0.05 vs controls, # p<0.005 vs obese subjects with IR

Figure 2. TNF alpha, IL-6, adiponectin serum concentrations in obese and control subjects

* p<0.001 vs controls; # p<0.005 vs obesities with IR

Figure 3. ACE gene expression after 18h Ang stimulation vs Insulin levels

Slope -8.050 ± 2.682 $r^2 = 0.4503$ p = 0.012

Figure 4. relationship between IL 6 and insulin levels in the group of obese with hsCRP > 3.

Spearman's rho -0.89 p=0.003

Figure 5. hsCRP and insulin levels in the obese group with low grade inflammation and IR and in the group of obese group without low grade inflammation and without IR.

hsCRP >3: slope -6.014 ± 1.752 $r^2 = 0.4419$ p=0.01

hsCRP ≤3 ns

