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ORIGINAL ARTICLE

Effect of antihypertensive treatments on insulin signalling in lympho-monocytes of essential hypertensive patients: A pilot study

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Abstract

It was previously demonstrated that metabolic syndrome in humans is associated with an impairment of insulin signalling in circulating mononuclear cells. At least in animal models of hypertension, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) may correct alterations of insulin signalling in the skeletal muscle. In the first study, we investigated the effects of a 3-month treatment with an ARB with additional PPARγ agonist activity, telmisartan, or with a dihydropyridine calcium channel blocker, nifedipine, on insulin signalling in patients with mild–moderate essential hypertension. Insulin signalling was evaluated in mononuclear cells by isolating them through Ficoll–Paque density gradient centrifugation and protein analysis by Western Blot. An increased expression of mTOR and of phosphorylated (active) mTOR (p-mTOR) was observed in patients treated with telmisartan, but not in those treated with nifedipine, while both treatments increased the cellular expression of glucose transporter type 4 (GLUT-4). We also investigated the effects of antihypertensive treatment with two drug combinations on insulin signalling and oxidative stress. Twenty essential hypertensive patients were included in the study and treated for 4 weeks with lercanidipine. Then they were treated for 6 months with lercanidipine + enalapril or lercanidipine + hydrochlorothiazide. An increased expression of insulin receptor, GLUT-4 and an increased activation of p70S6K1 were observed during treatment with lercanidipine + enalapril but not with lercanidipine + hydrochlorothiazide. In conclusion, telmisartan and nifedipine are both effective in improving insulin signalling in human hypertension; however, telmisartan seems to have broader effects. The combination treatment lercanidipine + enalapril seems to be more effective than lercanidipine + hydrochlorothiazide in activating insulin signalling in human lympho-monocytes.

Key Words: *Insulin signalling, Telmisartan, nifedipine, lercanidipine, enalapril hydrochlorothiazide, mTOR, GLUT-4, insulin lymphocites, monocytes*

Introduction

Insulin resistance is defined as insulin inability to stimulate muscle glucose uptake and disposal (1) and is a common feature of obesity, diabetes mellitus and essential hypertension (2–5). In particular, the skeletal muscle of hypertensive patients has a diminished insulin-stimulated glucose uptake (6,7). However, the evaluation of insulin signalling *in vivo* is not an easy task.

In the past few years, a method that allows the study of insulin signalling in human peripheral mononuclear cells was developed (8). This method has some relevant advantages, since it is relatively easy to perform and repeat, it avoids the pain or discomfort related to muscle biopsies, and it can be useful to assess the effects of interventions with specific therapeutic strategies, including drugs (8). Using this approach, we have observed that insulin signalling was significantly impaired in patients with metabolic syndrome, as confirmed by significantly reduced molecular concentrations serine/threonine-kinase mTOR (the mammalian target of

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rapamycin) and its downstream effectors p70-S6K1 and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) (8). In addition, we also analysed the molecules upstream mTOR involved in cellular insulin signalling, such as concentrations of insulin receptors and total insulin receptor substrate-1 (IRS-1): a significant reduction in insulin receptors was observed in patients with metabolic syndrome compared with controls while IRS-1 concentrations were similar between patients and controls (8).

mTOR is a serine/threonine kinase protein that plays a key role in insulin signalling and may be considered an important molecule of intracellular signalling. It lies at the centre of the metabolic pathway and operates in parallel to the cAMP pathway (9). Accordingly, mTOR influences the energy metabolism, protein synthesis, cell cycles and reparative processes including anti-apoptotic effects, which are fundamental for cell life span. Furthermore, mTOR regulates the expression of adhesion molecules and pro-survival signals in both circulating and endothelial cells influencing blood circulation and clotting (10,11). Clinical data show that inhibited mTOR, with specific inhibitors such as serolimus or everolimus after kidney transplantation, significantly increase the presence of *de novo* thrombotic microangiopathy with artery lesion characterized by intimal cell proliferation, necrosis and narrowed lumen (10). Complete withdrawal of mTOR inhibitors leads to clinical improvement in many cases (10). The increased incidence of vascular thrombosis when mTOR inhibitors are used and the improvement of micro-angiopathy when these drugs are withdrawn suggest the important role of mTOR in regulating vascular functions (11,12).

Several studies in humans have demonstrated that anti-hypertensive treatment with drugs that inhibit the renin–angiotensin system, namely angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB), may reduce new cases of diabetes mellitus (13). This protective action may be explained by effects on insulin signalling, with consequent improvement of insulin sensitivity. Munoz et al. (14) demonstrated that long-term selective angiotensin II blockade by irbesartan improves insulin signalling and is associated with decreased insulin receptor Ser994 phosphorylation in the liver of obese Zucker rats, an animal model of human metabolic syndrome. More recently we observed that changes in insulin signalling occur in the skeletal muscle but not in the heart of untreated spontaneously hypertensive rats (SHR) (15). In this study expression of insulin receptor, of IRS-1, mTOR, as well as of the of glucose transporter 4 (GLUT-4), were investigated. In the skeletal muscle, insulin signalling was restored by antihypertensive treatment with the ARB olmesartan, while the ACE inhibitor enalapril was less effective (15). Effective anti-hypertensive treatment with olmesartan or enalapril was associated with a prevention of microvascular rarefaction (15). There are, therefore, several reasons to support the hypothesis that drugs blocking the renin–angiotensin system may improve insulin signalling also in humans. On these bases, we considered it worthwhile to investigate the effects of different therapeutic approaches on insulin signalling in patients with essential hypertension, using a relatively simple and non-invasive method that allows the evaluation of involved proteins in blood lymphocytes.

We decided to subdivide our therapeutic approach into two studies: in the first study two monotherapies were compared, and in the second study two combination treatments were investigated, considering the expanding role of fixed or free drug combinations in the treatment of hypertension (16).

Patients and methods

Study 1

We investigated the effects of a 3-month treatment with an ARB with additional PPARγ agonist activity, telmisartan or with a dihydropyridine calcium channel blocker, nifedipine, possibly possessing antioxidant properties, on insulin signalling in patients with mild–moderate essential hypertension. Twelve patients were included in the study; six were treated with telmisartan (20–80 mg/day) and six with nifedipine in a slow-release formulation (20–60 mg/day). Patients were recruited from subjects admitted to outpatient clinics. Insulin signalling was evaluated, before and after antihypertensive treatment, by separating the mononuclear cells through the Ficoll–Paque density gradient centrifugation and protein analysis by Western Blot as described below.

We evaluated the expression of mTOR and of phosphorylated (active) mTOR (p-mTOR) as well as of insulin receptor and GLUT-4. In addition, demographic, haemodynamic and biochemical measurements were performed, including body mass index (BMI), blood pressure, fasting glucose, total cholesterol, triglycerides, low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol, and creatinine.

Study 2

We investigated the effects of antihypertensive treatment with two drug combinations on insulin signalling and oxidative stress. Twenty essential hypertensive patients were included in the study and treated for 4 weeks with lercanidipine 20 mg per day orally.

Then, patients were randomized to receive lercanidipine 20 mg per day + enalapril (up to 20 mg per day, $n = 10$) or lercanidipine 20 mg per day +

hydrochlorothiazide (up to 25 mg per day, $n = 10$) for 6 months. The dose of enalapril and hydrochlorothiazide was up-titrated if blood pressure was not at target (140/90 mmHg), starting from 10 mg of enalapril/12.5 mg hydrochlorothiazide.

Investigations were performed in basal condition, after 4 weeks of monotherapy with lercanidipine, and at the end of the combination treatment.

Anthropometric, haemodynamic and biochemical variables were evaluated or measured, including BMI, blood pressure, fasting glucose, total cholesterol, triglycerides, LDL- and HDL-cholesterol, serum uric acid, and creatinine.

Evaluation of circulating inflammatory markers and oxidative stress

Blood samples were collected between 08:00 and 09:00 h while participants were in a fasting state. After blood collection, plasma and serum were frozen in aliquots at −80°C immediately after centrifugation (4°C, 3000 rpm for 10 min). Circulating levels of C-reactive protein (CRP, Bender MedSystems, Austria, Europe), proinflammatory cytokines interleukin-6 (IL-6) and interleukin-18 (IL-18), macrophage chemotactic factor-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), and soluble Inter-Cellular Adhesion Molecule 1 (sICAM-1) (Bender MedSystems, Austria, Europe) were measured in plasma by ELISA technique following the directions of the supplier company. Total antioxidant power (AOP, Oxford Biomedical Research, MI, USA), malonyldialdehyde (MDA) and lipid peroxidation (LPO) (OxisResearch, CA, USA) were measured in plasma using spectrophotometric assay following the directions of the supplier company. Further details about the methods used are reported in ref. (17).

Isolation of human peripheral mononuclear cells and evaluation of proteins involved in insulin signalling

We evaluated the expression of insulin receptor, GLUT-4, IRS-1, p70S6K, AMP-kinase (AMPK), protein kinase AKT-1, 4E-BP1 and mTOR, all proteins involved with various roles in insulin signalling (10,18).

Data were normalized for tubulin expression (insulin receptor, GLUT-4) or for the expression of the non-phosphorylated inactive form (phosphorylated active p70-S6K, AMPK, AKT-1, 4E-BP1 and mTOR and phosphorylated inactive IRS-1).

Human peripheral mononuclear cells were obtained by Ficoll–Paque density gradient centrifugation as previously described (19).

Antibody against mTOR was obtained from Sigma-Aldrich (Milano, Italy). Anti-p-mTor, p-p70-S6K1, p-4E-BP1, p-serine-636/639-IRS-1 and anti-4E-BP1 were obtained from Cell Signalling Technology (Danvers, MA, USA). Anti-IRS-1 was obtained from Upstate (Charlottesville, VA, USA). Anti-AMPK, anti-GLUT4 and anti-Insulin Receptor alpha were from Santa Cruz Biotechnology (Heidelberg, Germany).

Total proteins were extracted from lymphomonocytes in lysis buffer (50 mM Tris.Cl pH 7.8, 1% Triton X100, 0.1% sodium dodecyl sulphate (SDS), 250 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM NaPPi, 2 mM Na_3VO_4, 1 mM PMSF). The crude lysate was centrifuged at 16 000*g*, the supernatant was recovered and assayed for protein concentration by the Bradford Assay (Bio-Rad Laboratories, Milano, Italy). Protein extracts were run on a 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) for IRS-1, *p*-serine 636/639-IRS-1, Insulin Receptor alpha and mTOR or 12% SDS–PAGE for p70-S6K1, p-p70-S6K1, 4E-BP1 and transferred onto a PVDF membrane (Millipore, Milano, Italy). The membranes were stained with Ponceau Red (Sigma-Aldrich, Milano, Italy) in order to check for the transfer and were blocked at room temperature for 2 h with 10% non-fat dry-milk in TBST containing 0.1% Tween20. After this, the blots were washed briefly and incubated with primary antibodies directed either against the phosphorylated forms 1:1000 overnight at 4°C) and against the others 1:500 O/N at 4°C, diluted with 5% non-fat milk in TBST 0.1% Tween20.

The membranes were then washed three times for 10 min with TBST. Then, they were incubated for 1 h at room temperature, with anti-rabbit or anti-mouse (depending on the primary antibody) HRP-conjugated secondary antibody (Bio-Rad Laboratories, Milano, Italy) diluted 1/2000 in TBST containing 5% non-fat milk. The membranes were washed three times for 10 min, incubated in SuperSignal West Pico (Thermo Fisher Scientific, Erembodegem, Belgium) chemo-luminescent substrate and exposed to ChemiDoc XRS System (Bio-Rad Laboratories, Milano, Italy). The optical densities of blot bands were finally determined using a computer-assisted densitometer (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2006). For further information about the method, see ref. (8).

The study protocols were approved by the Ethics committee of our institution (University of Brescia Medical School), and informed consent was obtained from each participant. All procedures were in accordance with institutional guidelines.

Statistical approach

Results are expressed as means ± standard deviation (SD). Comparison of continuous variables in the clinical study was performed by Student paired

or unpaired *t*-test, as appropriate. The statistical significance was set at the conventional level of 5%. All variables investigated were normally distributed. Being a hypothesis-generating study, no corrections for multiple comparisons were made.

According to standard deviations observed in our previous study (8), a total sample size of 20 patients randomized in a 1:1 ratio achieved a power greater than 80% to detect a statistically significant difference in GLUT-4 and mTOR with a two-tailed type I error equal to 0.05.

Results

Study 1

Demographic variables are reported in Table I. There was no difference, between groups or versus baseline, observed in the expression of insulin receptor (Figure 1). An increased expression of phosphorylated (active) mTOR (p-mTOR) was observed in patients treated with telmisartan, but not in those treated with nifedipine, while both treatments increased the cellular expression of GLUT-4 (Figure 1). The two treatment groups were unbalanced for basal values of mTOR.

Study 2

Demographic variables are reported in Table II.

A modest reduction in circulating levels of IL 18, CRP and MCP-1 was observed after treatment with lercanidipine alone; however, differences reached statistical significance only in patients that subsequently were randomized to lercanidipine + enalapril (Table III). Differences vs basal values persisted (IL 18, MCP-1) or further improved (CRP) after treatment with lercanidipine + enalapril but not with lercanidipine + hydrochlorothiazide (Table III). No change was observed for the remaining markers of inflammation/oxidative stress with any treatment (Table III).

An increased expression of insulin receptor, GLUT-4 and an increased activation of p70-S6K1 were observed during treatment with lercanidipine + enalapril but not with lercanidipine + hydrochlorothiazide (Figure 2, Table IV). No difference was observed between groups or versus baseline for other variables investigated (Table IV). Changes after treatment with lercanidipine alone were of modest entity (Figure 2, Table IV).

Discussion

Our results suggest for the first time that the evaluation of insulin signalling in blood cells such as lymphocytes, using a relatively simple and easily repeatable procedure, may be safely applied to the evaluation of the metabolic effects of antihypertensive treatment and that different drugs/drug combinations may have different effects on proteins involved in insulin signalling.

There are many reasons suggesting that proteins involved in intracellular insulin signalling might represent an interesting and clinically useful therapeutic target. In fact, it was previously demonstrated that mTOR, which regulates replacement of damaged blood and endothelial cells with consequent maintenance of vasculature integrity and potential regulation of thrombotic phenomena, as well as other molecules involved in the intracellular insulin signalling, are significantly altered in patients with clinical features of the metabolic syndrome (8).

Interestingly enough, Morisco et al. also demonstrated the presence of a cross-talk between β-adrenergic stimulation and insulin signalling through AKT, suggesting that there is an interrelationship between the activation of the sympathetic nervous system and insulin signalling including AKT, which influences mTOR function

Table I. Demographic haemodynamic and humoral data in study 1.

	All patients $n = 12$		Nifedipine $n = 6$		Telmisartan $n = 6$	
	Basal	After 3 months	Basal	After 3 months	Basal	After 3 months
Age (years)	53.08 ± 11.3		48.5 ± 18.8		57.6 ± 8.1	
Gender (M/F)	6/6		1/5		5/1	
BMI (kg/m^2)	27.4 ± 4.8		28.0 ± 5.1		26.8 ± 4.98	
SAP (mmHg)	143.5 ± 9.8	128.0 ± 12.0***	141.0 ± 10.1	127.1 ± 10.1**	146.1 ± 9.7	128.8 ± 14.5*
DAP (mmHg)	88.7 ± 8.3	82.5 ± 7.5**	92.1 ± 5.6	87.5 ± 6.1	85.3 ± 9.7	77.5 ± 5.2*#
HR (beats/min)	75.6 ± 12.0	75.6 ± 8.4	74.6 ± 11.7	77.3 ± 4.1	77.6 ± 13.6	74.0 ± 11.5
Serum glucose (mg/dl)	120.1 ± 50.8	116.4 ± 38.2	125.5 ± 63.6	113.8 ± 42.5	114.8 ± 39.5	119.0 ± 37.2
Serum creatinine (mg/dl)	0.8 ± 0.13	0.76 ± 0.11	0.78 ± 0.13	0.72 ± 0.09	0.87 ± 0.12	0.80 ± 0.12
Total cholesterol (mg/dl)	207.5 ± 47.7	201.0 ± 36.1	198.6 ± 49.2	198.3 ± 41.0	216.3 ± 49.1	203.8 ± 34.2
LDL-cholesterol (mg/dl)	128.8 ± 37.6	123.5 ± 26.1	119.1 ± 34.0	122.0 ± 28.5	138.5 ± 41.6	125.0 ± 26.1
HDL-cholesterol (mg/dl)	58.8 ± 18.1	57.4 ± 17.6	57.1 ± 19.7	53.3 ± 14.9	59.0 ± 18.1	61.5 ± 20.5
Triglycerides (mg/dl)	103.1 ± 43.2	100.5 ± 49.3	112.3 ± 47.9	114.8 ± 66.0	94.0 ± 40.2	86.1 ± 22.4

BMI, body mass index; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR, hear rate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs basal; #$p < 0.05$ vs nifedipine.

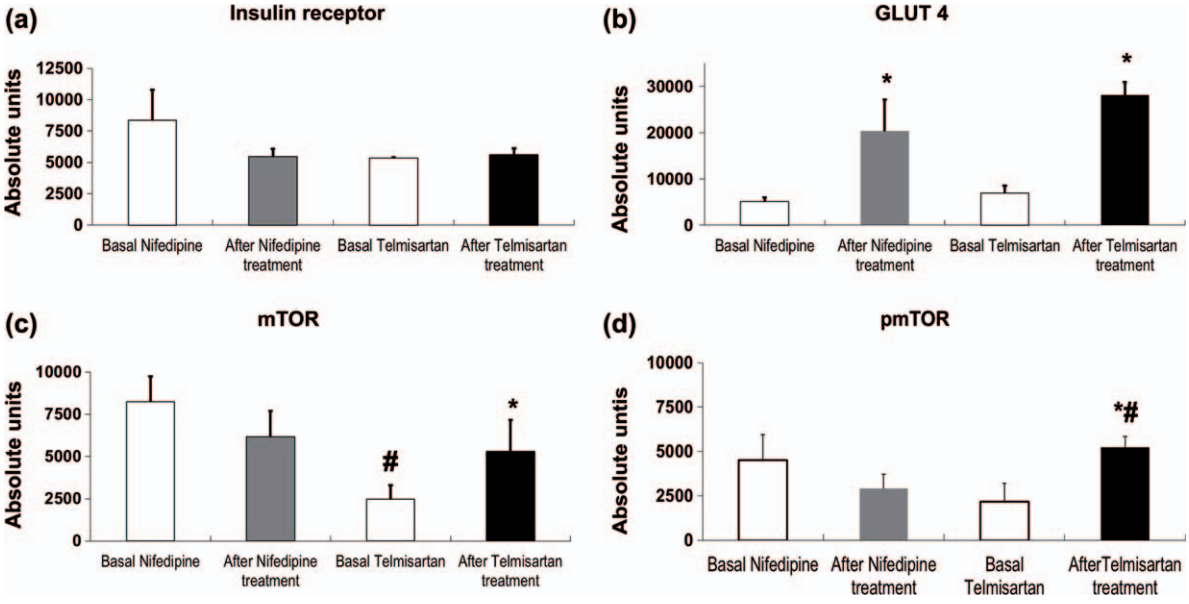

Figure 1. Intracellular concentrations of insulin receptor (panel a), GLUT-4 (panel b), mTOR (panel c) and phosphorylated (active) mTOR: p-mTOR, (panel d) before and after treatment with nifedipine or telmisartan, evaluated by Western blot (absorbance units). $p < 0.05$ vs basal values; $^{\#}p < 0.05$ vs nifedipine.

(20). The role of mTOR and cross-talk with inflammatory and sympathetic systems and insulin signalling are very new and interesting topics and deserve further investigations in order to understand the molecular pathophysiology responsible for the increased cardiovascular disease associated with hypertension and the metabolic syndrome. Moreover, we have recently shown that maintenance of cellular mTOR function by anti-hypertensive drugs improves insulin signalling by increasing GLUT-4 expression and prevents micro-vascular rarefaction in SHRs with insulin resistance (15). This effect was independent of the reduction of blood pressure, but was mTOR-related (15). As previously mentioned, we also found impairment of intracellular insulin signalling in patients with metabolic syndrome (8).

Table II. Demographic data in the different groups of patients enrolled in study 2.

	Group 1: Basal ($n = 10$)	Group 2: Basal ($n = 10$)	Group 1: 4 weeks, lercanidipine alone ($n = 10$)	Group 2: 4 weeks, lercanidipine alone ($n = 10$)	Group 1: Lercanidipine + enalapril, 24 weeks ($n = 10$)	Group 2: Lercanidipine + hydrochlorothiazide, 24 weeks ($n = 10$)
Age (years)	58.1 ± 6.32	49.3 ± 11.76	–	–	–	–
Gender (M/F)	9/1	7/3	–	–	–	–
BMI (kg/m^2)	27.6 ± 3.04	26.5 ± 3.30	27.6 ± 3.04	26.5 ± 3.30	27.6 ± 3.04	26.5 ± 3.30
SBP (mmHg)	153.7 ± 9.43	158.0 ± 8.67	146.5 ± 11.00*	148.9 ± 8.52**	136.0 ± 15.34***#	133.2 ± 10.05***###
DBP (mmHg)	94.7 ± 7.15	96.6 ± 12.3	92.7 ± 5.38*	92.5 ± 6.04	84.8 ± 8.01***##	82.2 ± 6.46***###
Serum glucose (mg/dl)	92.9 ± 10.24	92.8 ± 6.07	82.2 ± 29.28	94.2 ± 8.34	94.4 ± 6.55	96.8 ± 7.97
Serum creatinine (mg/dl)	0.85 ± 0.15	0.92 ± 0.16	0.83 ± 0.17	0.91 ± 0.16	0.82 ± 0.15	0.89 ± 0.17
Triglycerides (mg/dl)	152.6 ± 121.98	142.9 ± 95.25	117.1 ± 49.91	141.0 ± 96.56	130.3 ± 81.47	150.6 ± 93.23
Total cholesterol (mg/dl)	221.5 ± 27.14	204.2 ± 36.05	202.8 ± 27.00	207.5 ± 30.29	204.7 ± 29.74	213.8 ± 35.29
LDL-cholesterol (mg/dl)	134.4 ± 20.85	120.7 ± 27.16	117.2 ± 33.18	120.3 ± 25.71	121.7 ± 27.59	128.0 ± 26.27
HDL-cholesterol (mg/dl)	55.8 ± 18.45	54.4 ± 17.44	58.8 ± 20.21	55.7 ± 16.51	56.9 ± 19.85	55.6 ± 12.99
Serum uric acid (mg/dl)	5.63 ± 1.52	4.76 ± 1.65	6.1 ± 1.16	5.8 ± 1.57	5.7 ± 1.28$^{\#}$	6.3 ± 2.02*#

BMI, body mass index; SBP, systolic blood pressure; DBP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Group 1: randomized to lercanidipine + enalapril. Group 2: randomized to lercanidipine + hydrochlorothiazide. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs basal; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs lercanidipine alone 4 weeks.

Table III. Intracellular expression of proteins involved in insulin signalling in the different group of patients enrolled in study 2.

	Group 1: Basal ($n = 10$)	Group 2: Basal ($n = 10$)	Group 1: 4 weeks, lercanidipine alone ($n = 10$)	Group 2: 4 weeks, lercanidipine alone ($n = 10$)	Group 1: Lercanidipine + enalapril, 24 weeks ($n = 10$)	Group 2: Lercanidipine + hydrochlorothiazide, 24 weeks ($n = 10$)
mTOR	1.31 ± 0.57	1.41 ± 1.46	1.40 ± 0.59	1.36 ± 0.95	1.34 ± 0.57	1.40 ± 0.70
AMPK	1.11 ± 0.68	0.95 ± 0.62	1.11 ± 0.51	0.65 ± 0.64	0.91 ± 0.35	0.92 ± 0.93
Akt-1	0.95 ± 0.55	0.76 ± 0.39	1.09 ± 0.60	0.72 ± 0.29	1.25 ± 0.55	0.84 ± 0.25
4-EBP1	1.67 ± 1.79	0.97 ± 1.37	1.56 ± 1.64	0.31 ± 0.54	1.37 ± 0.82	0.72 ± 0.89
IRS-1	0.73 ± 0.36	0.81 ± 0.40	0.87 ± 0.23	0.84 ± 0.65	0.98 ± 0.26	1.01 ± 0.90
p70S6K	0.96 ± 0.67	1.14 ± 0.93	1.25 ± 0.71	1.20 ± 0.93	1.58 ± 0.88*	1.14 ± 0.43
Insulin receptor	1.02 ± 0.63	0.80 ± 0.41	1.02 ± 0.52	1.19 ± 0.95	1.38 ± 0.50#	1.01 ± 0.62
GLUT-4	0.44 ± 0.15	0.54 ± 0.42	0.53 ± 0.40	0.46 ± 0.18	1.23 ± 0.67**#	0.57 ± 0.41

Group 1: randomized to lercanidipine + enalapril; Group 2: randomized to lercanidipine + hydrochlorothiazide. Insulin receptor and GLUT-4: data are normalized for tubulin expression; p70-S6K1, mTOR, AKT, EBP1, IRS-1: data are normalized for the expression of the non-phosphorylated (inactive) form. *$p < 0.05$, **$p < 0.01$ vs basal values, #$p < 0.05$ vs lercanidipine alone (4 weeks).

Indeed, insulin signalling is a complex phenomenon where mTOR may play a fundamental role (10,21). In detail, insulin binding to its specific receptor leads to the autophosphorylation of the transmembrane β receptor sub-units and tyrosine phosphorylation of IRS-1 after their recruitment to the cell membrane. When IRS-1 is activated, it stimulates GLUT 4, with consequent regulation of glucose and lipid intracellular metabolism. In addition, activated IRS-1 modulates the phosphoinositide 3-kinase (PI3K) that in turn indirectly stimulates the activity of mTOR (22). As discussed before, mTOR is a central regulator of cellular responses to hormones, growth factors and nutrients (10,23). Current understanding of insulin signalling considers IRS-1 a key regulatory protein in this cascade and thus for mTOR activation. The main cellular molecular mechanism of insulin desensitization, with consequent insulin resistance presents in patients with clinic features of the metabolic syndrome involves increased serine phosphorylation and decreased tyrosine phosphorylation of IRS-1. This is true in type 2 diabetic patients as well as in experimental models of insulin resistance. Phosphorylation of the tyrosine residues 608 on IRS-1 after insulin stimulation is necessary for propagation of the signal with consequent active-mTOR expression. IRS-1 function is also negatively regulated by other circulating molecules found in the metabolic syndrome such as catabolic hormones and inflammatory molecules (20). Although little is known about the intracellular molecular mechanisms present in metabolic syndrome, it is possible that some alteration in intracellular signalling may be important for the development of vascular damage (24).

Hypertension is one of the key clinical signs of the metabolic syndrome, together with obesity, hypertension and alteration of glucose or lipid metabolism (25). Circulating molecules such as stress hormones and inflammatory cytokines may play a relevant role in the development of target organ damage in hypertension (26,27). Molecular alterations can be therefore used as biomarkers of this disease and its evolution. In our study, we not only investigated mTOR, but also its downstream effectors p70-S6K and 4E-BP1, which stimulate anabolic pathway and other fundamental biochemical pathways such as the production of adhesion molecules; in addition they may influence the replacement of damaged cells and, in general, cell survival (8,10,23). In study 1 we have observed that telmisartan was more effective than nifedipine in stimulating the intracellular expression of mTOR, also in its activated form. Since these preliminary data had to be confirmed in a larger study, we investigated also the possible effect of different combination treatments on proteins involved in insulin signalling. The combination treatment lercanidipine + enalapril was more effective than lercanidipine + hydrochlorothiazide in activating insulin signalling in human lympho-monocytes, possibly due to a more marked antioxidant/anti-inflammatory effect. The reasons for these differences could be related to intrinsic or pharmacodynamic properties of the drugs. Dihydropyridine calcium channel blockers may possess antioxidant properties (28,29), while ACE inhibitors and ARB, together with antioxidant and vasculoprotective effects due to the inhibition of angiotensin II mediated actions (15), may possess also anti-inflammatory properties (26,27). This might partially provide an explanation for the modest differences in the effects on insulin signalling observed in study 1, where an ARB and a calcium channel blocker were compared head to head. As previously mentioned, inhibition of the renin–angiotensin system may also directly improve insulin signalling by increasing expression of IRS-1 (14), although other indirect actions might also be involved (effects on oxidative stress, on PPARγ systems, on microvascular rarefaction, or on G proteins) (15,26).

It should also be mentioned that it is highly plausible that calcium entry blockade, especially when associated with inhibition of the renin–angiotensin system, may prove more effective, compared with the association with a diuretic, that is to say a drug

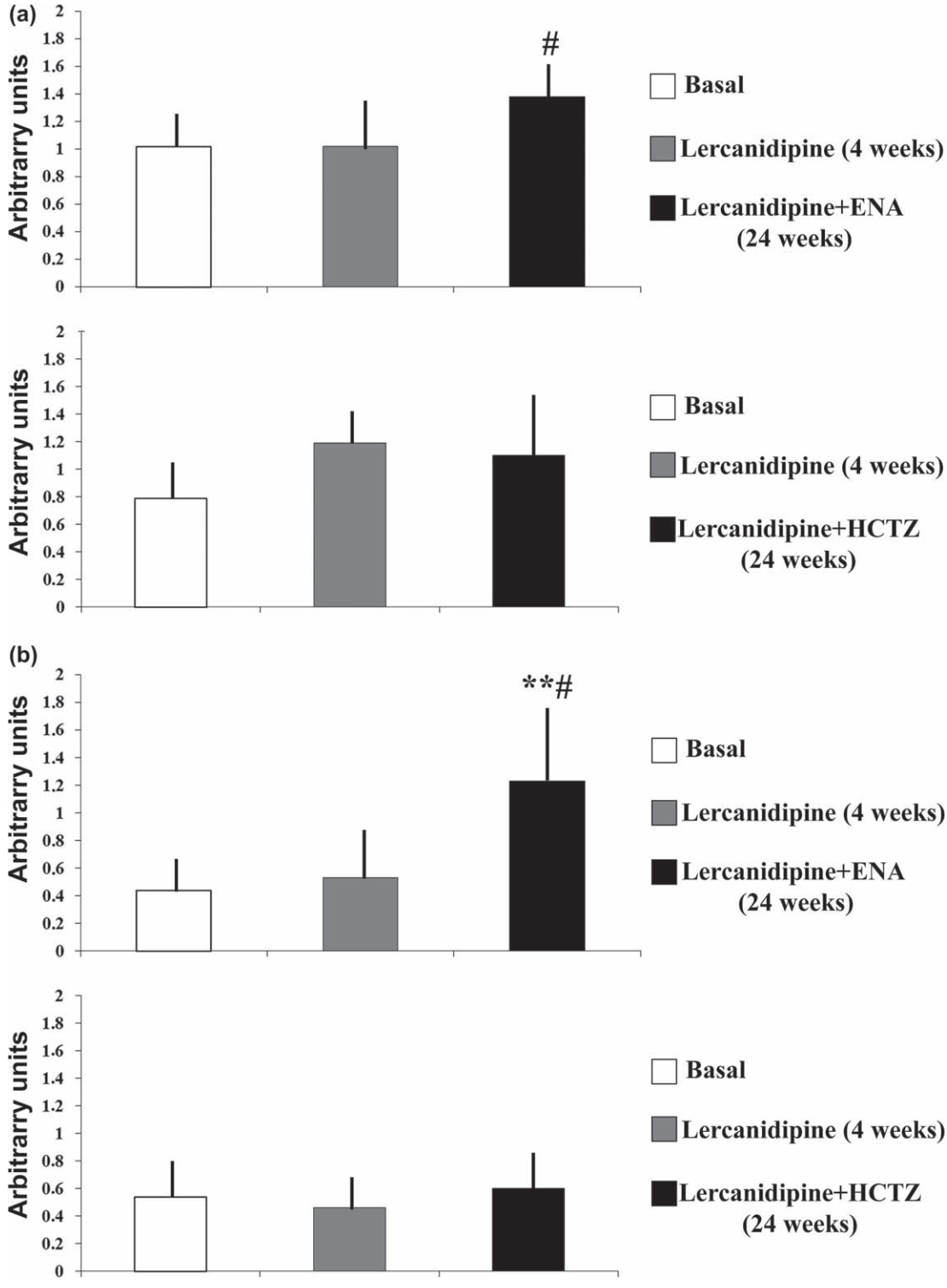

Figure 2. Intracellular concentrations of insulin receptor (panel a), GLUT-4 (panel b) and p70-S6K1, (panel c) before and after treatment with lercanidipine alone or combination treatment with lercanidipine + enalapril/lercanidipine + hydrocholorothiazide, evaluated by Western blot. Insulin receptor and GLUT-4: data are normalized for tubulin expression; p70-S6K1: data are normalized for the expression of the non-phosphorylated (inactive) form. $^{*}p < 0.05$, $^{**}p < 0.01$ vs basal values, $^{\#}p < 0.05$ vs lercanidipine alone (4 weeks).

possibly possessing undesirable metabolic properties (30,31), when effects on intracellular insulin signalling are concerned. Cellular findings linked to glucose metabolism do not translate always into clinical conditions. Blockade of the renin–angiotensin system with ACE inhibitors or ARB was indeed associated with a reduced incidence of new onset diabetes (13). No difference between the two drug classes should be expected in this regard, since they probably share most of the specific effects, as suggested by head to heads comparisons (e.g. ONTARGET study) (32). The TRANSCEND study, in a peculiar setting of patients with heart failure, failed to demonstrate an advantage of telmisartan over placebo in terms of

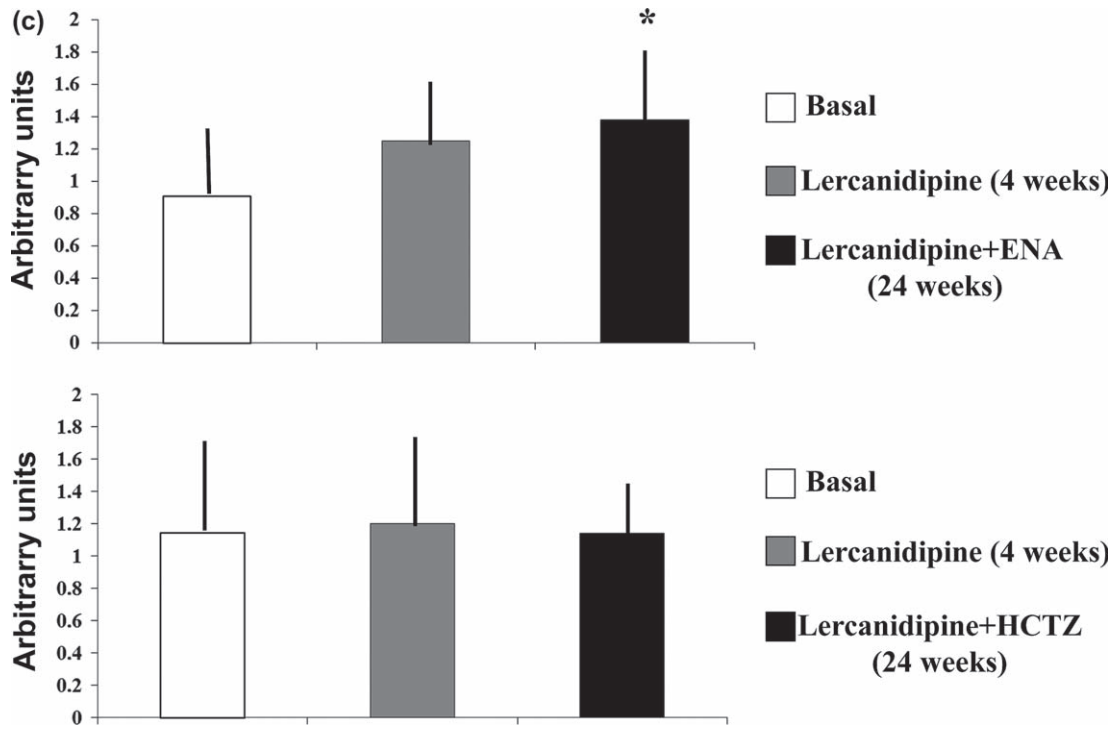

Figure 2.(*Continued*).

glycaemic control (33). Many large-scale studies in essential hypertension have shown no additional benefit of ACE inhibitors compared with diuretics, apart from the ANBP2 study (34), in which treatment with ACE inhibitor in older subjects appears to lead to better outcomes than treatment with diuretic agents. However, as far as association treatments are concerned, the ACCOMPLISH trial has demonstrated that the combination between an ACE inhibitor and a dihydropyridine calcium channel blocker was superior to the ACE inhibitor–hydrochlorothiazide combination in reducing cardiovascular events in patients with hypertension (35). The benefits were particularly evident in patients with diabetes mellitus (35), thus supporting possible metabolic advantage of such a combination.

In conclusion, different drugs/drug combinations may have different effects on insulin signalling, and this might have clinical consequence in terms of vasculoprotection/incidence of new cases on diabetes mellitus in patient with essential hypertension. An ARB seems to have a modest advantage over a

Table IV. Circulating indices of oxidative stress/inflammation in the different groups of patients enrolled in study 2.

	Group 1: Basal ($n=10$)	Group 2: Basal ($n=10$)	Group 1: 4 weeks, lercanidipine alone ($n=10$)	Group 2: 4 weeks, lercanidipine alone ($n=10$)	Group 1: Lercanidipine+ enalapril, 24 weeks ($n=10$)	Group 2: Lercanidipine+ hydrochlorothiazide, 24 weeks ($n=10$)
Total antioxidant power (μM)	0.46 ± 0.091	0.42 ± 0.10	0.41 ± 0.09	0.041 ± 0.08	0.43 ± 0.050	0.41 ± 0.06
LPO (μM)	2.07 ± 0.70	2.44 ± 0.46	2.12 ± 0.73	1.98 ± 0.62	3.26 ± 1.98	2.74 ± 1.86
MDA (mM)	145.15 ± 54.33	108.46 ± 44.3	145.85 ± 103.1	181.26 ± 144.2	246.21 ± 229.5	102.48 ± 34.33
MCP-1 (pg/ml)	1012 ± 110	1078 ± 401	853 ± 182*	1079 ± 41	865 ± 136**†	1204 ± 461
IL-6 (pg/ml)	11.52 ± 2.48	22.77 ± 10.98	13.01 ± 5.73	10.98 ± 2.57	11.12 ± 1.31	11.36 ± 1.19
IL-18 (pg/ml)	436.4 ± 87.81	402.4 ± 49.00	341.44 ± 106.4*	388.40 ± 62.78	400.8 ± 75.42*	423.24 ± 146.8
sICAM-1 (ng/ml)	244.12 ± 85.94	240.52 ± 58.2	211.47 ± 77.9	231.5 ± 49.4	226.02 ± 79.0	186.3 ± 48.1
sVCAM-1 (ng/ml)	887 ± 235	990 ± 246	994 ± 713	1062 ± 323	860 ± 229	1118 ± 545
TNF-α (pg/ml)	40.46 ± 3.56	40.2 ± 3.08	40.8 ± 3.05	41.0 ± 4.00	41.6 ± 3.19	44.6 ± 9.10
PAI-1 (ng/ml)	279.37 ± 90.27	294.90 ± 40.01	247.17 ± 86.83	309.89 ± 57.38	272.23 ± 61.70	303.67 ± 54.71
CRP (ng/ml)	1076 ± 755	690 ± 457	767 ± 514*	526 ± 472	456 ± 349*#	583 ± 543

LPO, lipid peroxidation; MDA, malonyldialdehyde; MCP-1, macrophage chemotactic factor-1; IL-6, interleukin-6; IL-18, interleukin-18; sICAM-1, soluble Inter-Cellular Adhesion Molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1; TNF-α, tumour necrosis factor-α; PAI-1, plasminogen activator inhibitor-1; CRP, C-reactive protein. Group 1: randomized to lercanidipine + enalapril; Group 2: randomized to lercanidipine + hydrochlorothiazide. *$p<0.05$ **$p<0.01$, vs basal; #$p<0.05$ vs lercanidipine alone 4 weeks: †$p<0.05$ vs lercanidipine + hydrochlorotiazide.

dihydropyridine calcium channel blocker in this regard, while the combination of ACE inhibitor plus a dihydropyridine calcium channel seems to be more effective than the combination of an ACE inhibitor plus a thiazide diuretic in terms of expression of proteins involved in insulin signalling. This may partially help us to explain epidemiological data about the incidence of new cases of diabetes mellitus during antihypertensive treatment (13).

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