

# **Acipimox reduces circulating levels of insulin and associated pro-atherosclerotic neutrophilic inflammation in metabolic syndrome**

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## **Abstract**

**Aims:** Metabolic syndrome is a pro-atherosclerotic condition clustering cardiovascular risk factors, including glucose and lipid profile alterations. Metabolic mechanisms favoring atherosclerotic inflammation underlying this disease remain elusive. Here, we investigated the potential role of the anti-lipolytic drug Acipimox on neutrophilic inflammation in metabolic syndrome. **Materials and Methods:** 500 mg of Acipimox was orally administered to metabolic syndrome patients (n=11) or healthy controls (n=3). Serum and plasma were collected before (time 0) Acipimox administration as well as 2h-5h after to assess metabolic and hematologic parameters. *In vitro*, the effects of incubation with metabolic syndrome serum were assessed on human neutrophil migration towards the CCL3 and associated JNK 1/2 activation. **Results:** 2h-5h after Acipimox administration, a significant reduction of circulating levels of insulin and nonesterified fatty acid (NEFA) was shown in metabolic syndrome patients. At time 0 and 2h after Acipimox assumption, metabolic syndrome serum increased neutrophil migration to CCL3 as compared to healthy controls. At these time points, serum-induced neutrophil migration to CCL3 positively correlated with circulating levels of insulin and NEFA. 3h-5h after Acipimox administration, serum-induced neutrophil locomotion was abrogated. Insulin immunodepletion blocked serum-induced neutrophil migration towards CCL3 and associated JNK1/2 activation. Although mRNA expression of Acipimox receptor (GPR109) was shown in human neutrophils, 5-500  $\mu$ M Acipimox did not affect insulin-induced neutrophil migration. **Conclusions:** Results suggest that Acipimox inhibited neutrophil pro-atherosclerotic functions in metabolic syndrome through the reduction of circulating levels of insulin.

## **Introduction**

Alterations in lipid profile have been associated with high circulating levels and resistance to insulin, a condition favoring atherogenesis and acute cardiovascular events [1-3]. Chronic exposure to high plasma levels of free fatty acids (FFAs) has been shown as a key “lipotoxic” event, inducing in first phases hyperinsulinemia till final pancreatic  $\beta$  cell failure [1]. On the other hand, FFAs potently influence insulin-mediated functions, by directly interfering with insulin receptor activation and intracellular signalling [1]. The increase of plasma FFA levels has been shown as mediated by both prolonged fasting (sustained lipolysis) and excessive fat intake [4]. The physiological FFA modifications are chronically exaggerated in metabolic syndrome causing the final development of insulin resistance. Despite not necessary for the diagnosis of metabolic syndrome [5], insulin resistance can initiate a dangerous vicious circle in these patients, involving inflammation and hypercoagulability, which further favor atherogenesis [6, 7]. Thus, pharmacologic improvement in FFA concentrations could be a very promising approach to reduce insulin resistance and associated cardiovascular risk [1]. Among several medications, the anti-lipolytic drug Acipimox has been shown to improve insulin resistance and oral glucose tolerance in obese subjects [8]. Acipimox (5-methylpyrazine-2-carboxylic acid-4-oxide) is a nicotinic acid derivative inhibiting lipolysis in adipose tissue [9]. More recently, Acipimox has been also shown to bind nicotinic acid receptor (GPR109) in other tissues [10]. This discovery suggested a potential direct pleiotropic activity of Acipimox on cell types governing atherosclerotic inflammation. The aim of the present study was to evaluate the effects of a single administration of Acipimox on metabolic parameters and neutrophil pro-atherosclerotic functions in metabolic syndrome. The direct role of Acipimox on the modulation of insulin levels and activities on atherosclerotic neutrophilic inflammation was also investigated.

## **Materials and methods**

### *Patients and study design*

We tested Acipimox treatment in a case-control study between December 2009 and June 2010 at a single hospital (San Martino Hospital) in Genoa (Italy). A case was defined as a patient with metabolic syndrome (diagnosed following NCEP ATP III criteria) [5]. Exclusion criteria were smoking habit, acute and chronic coronary artery and cerebrovascular disease, any cardiac arrhythmias, congestive heart failure (II, III and IV NHYA classes), liver or renal disorders or function abnormalities (other than microalbuminuria), acute and chronic infectious diseases, autoimmune and rheumatic diseases, cancer, endocrine diseases, inflammatory bowel diseases, anti-inflammatory medications, oral anticoagulant treatments, hormone, cytokine or growth factor therapies. Current medications (not modified in the 2 months prior to enrolment) of metabolic syndrome patients were diuretics (33%), angiotensin receptor blockers (18.2%), beta-blockers (9.1%), calcium channel blockers (9.1%). To avoid possible pharmacological interference, these medications were stopped during the week preceding Acipimox test. A control was defined as a healthy subject without clinical evidence of any disease and any pharmacological treatments. In healthy controls, blood, metabolic and inflammatory laboratory parameters were in the normal ranges. Metabolic syndrome patients and healthy controls were submitted to the same research protocol (Figure 1), approved by the Medical Ethics Committee of San Martino Hospital. The study was conducted in compliance with the Declaration of Helsinki. After a standard meal of 600 Kcal at 8.00 pm the day before Acipimox test, subjects were instructed to remain fasted and avoid any physical activity (from moderate to strong level) till the end of the test. Only water consumption was consented. The day of the test, at 8.00 am, a single dose (500 mg) of Acipimox (Farmitalia Carlo Erba, Milan, Italy) was orally administered to metabolic syndrome patients or healthy controls. Just before (time 0) and 2h-5h after Acipimox administration, serum and plasma were

collected to perform metabolic parameter measurements and neutrophil experiments (Figure 1).

#### *Systemic inflammatory marker detection*

Serum insulin levels were measured by colorimetric enzyme-linked immunosorbent assay (Millipore, St. Charles, MO), following manufacturer's instructions. Plasma nonesterified fatty acid (NEFA) levels were measured by enzymatic color test Wako NEFA C (Wako Chemicals GmbH, Neuss, Germany), following manufacturer's instructions. The lower limit of detection for insulin was 2 mU/l. NEFA kit ranged up to 56 mg/dl. Mean intra- and inter-assay coefficients of variation (CV) were below 5% for both parameters. Hematologic parameters were routinely measured. Glucose, triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol were routinely measured and expressed in mg/dl. The homeostasis model assessment (HOMA) index was calculated by using the formula described by Matthews and co-workers [11]:  $\text{insulin (mU/l)} \times [\text{glucose (mmol/l)} / 22.5]$ .

#### *Human primary neutrophil isolation and migration assay*

Neutrophils were isolated from human heparinized (from Roche [Milan, Italy]) venous blood from healthy volunteers (age 25–48 years old), after informed and written consent. The local ethical committee approved the investigation protocol, and it was conformed to the principles outlined in the Declaration of Helsinki. Human neutrophils were isolated by heparinized venous blood by dextran sedimentation followed by centrifugation on Ficoll–Hypaque (from Cedarlane Laboratories Ltd. [Ontario, Canada]) density gradient, as previously described [12]. Neutrophils were routinely >97% pure, as determined by morphologic analysis of cytopreparations stained with Giemsa (from Merck [Darmstadt, Germany]). Then, cells were

suspended in medium (Hanks' balanced salt solution [HBSS; EuroCLone, Wetherby West, UK] mixed with Dulbecco's PBS [EuroCLone; HBBS:PBS = 3:1] containing 1 mg/ml BSA [Sigma Chemical Co., St. Louis, MO] for migration assay.

#### *Insulin immunodepletion in metabolic syndrome serum*

Serum from metabolic syndrome patients was immunodepleted of insulin, as previously described for other factors [13, 14]. Serum (1 ml) was incubated with 2 µg of anti-human insulin Ab (Santa Cruz Biotechnology, Santa Cruz, CA) on a rotating wheel (overnight at 4°C) and then ultracentrifuged (10000 g, 45 min). Prior to immunodepletion, the anti-human insulin Ab was dialyzed against the buffer for 1 hour at 4°C, using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) to avoid a contamination of the serum samples with sodium azide. Immunoprecipitated insulin was removed and supernatants was collected and stored at -20°C. After immunodepletion, the insulin levels in metabolic syndrome sera were measured by ultrasensitive ELISA (Merckodia AB, Uppsala, Sweden) and found under the lower range limit (<0.15 mU/l).

#### *Modified Boyden chamber migration assay*

Neutrophils were pre-incubated 30 min with control migration medium alone or undiluted serum (normal or insulin-immunodepleted), obtained at different time points from metabolic syndrome patients and healthy subjects during Acipimox test. In selective experiments, neutrophils were pre-incubated for 1 hour in the presence or the absence of different concentrations (5-500 µM) of Acipimox (Sigma Co, St. Louis, MO). Then, after washing three times, cells were further incubated 30 minutes in the presence of migration medium or recombinant human insulin (300 mU/l, Lilly France SAS, France). Then, in both experiment series, cells were washed with migration medium and neutrophil migration towards control

medium, human recombinant CCL3 (R&D Systems, Minneapolis, MN), human recombinant CXCL8 (BioSource International, Camarillo, CA) was assessed in a 48-well microchemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD) using a 3- $\mu\text{m}$  pore size, 150- $\mu\text{m}$ -thick cellulose ester filter (Neuro Probe, Inc.). The lower wells of chemotaxis chamber were filled with control medium, 30 nM CCL3 or 1 nM CXCL8 and the upper wells with 50  $\mu\text{l}$  of neutrophil suspension ( $2 \times 10^6/\text{ml}$ ). After incubation (45 min, 37°C), the filters were removed, fixed and stained with Harris haematoxylin, dehydrated cleared with xylene and mounted in Eukitt (Kindler, Freiburg, Germany). Each condition was performed in duplicate. The distance (migration expressed in  $\mu\text{m}$ ) traveled by the leading front of cells was measured by a blinded observer at 1000x magnification. Data were expressed as migration towards control medium or chemokines ( $\mu\text{m}$ ) or, alternatively, net migration ( $\mu\text{m}$ , obtained subtracting locomotion to control medium from locomotion to chemokine). Each condition was performed three times isolating neutrophils from three different healthy donors.

#### *Western blot analysis*

After isolation, human neutrophils ( $1 \times 10^7$  cells/ml) were incubated at 37°C in a humidified atmosphere 5% CO<sub>2</sub> for 3 minutes in the presence or absence of chemotaxis medium, 300 mU/l recombinant insulin (Lilly France SAS), serum from a metabolic syndrome patient (MS #1), serum from second metabolic syndrome patient (MS #2), or the same sera immunodepleted of insulin (i-MS #1 and i-MS #2). Metabolic syndrome serum collected at time 0 (highest circulating insulin concentrations) was used. The incubations were stopped on ice and the cells were centrifuged at 4°C. After removing supernatants, the pellets were lysed in 400  $\mu\text{l}$  of Nonidet P40 buffer (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 10 mM NaF, 1% Nonidet P40, 10  $\mu\text{g}/\text{ml}$  glycerol, 1 mM phenylmethanesulphonyl-fluoride (PMSF), 10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  aprotinin, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>). Equal amounts of protein (30  $\mu\text{g}$ ) for each

sample were boiled in loading buffer (62.5 mM Tris-HCl pH 6.8, 0.75% SDS, 3.75% 2-mercaptoethanol, 8.75% glycerol and 0.025% bromophenol blue) and resolved by 10% SDS-polyacrylamide electrophoresis. Then, proteins were transferred on nitrocellulose membrane at 4°C for 45 minutes. After blocking 1 hour in 5% non-fat dry milk and washing with Tris-buffered saline/Tween 20 (TBS-T, containing 10 mM Tris-base pH 7.4, 154 mM NaCl and 0.05% Tween 20), membranes were incubated with appropriate dilution of anti-phospho-JNK 1/2 primary Ab (Santa Cruz Biotechnology), as well as corresponding secondary Ab. Blots were developed using the ECL system (Immobilion Western, Millipore, USA). Membranes were then stripped, reblocked and reprobed to detect total JNK 1/2 (Santa Cruz Biotechnology). Immunoblots were scanned and quantifications was carried out by Image Quant software version 3.3 (Molecular Dynamics, Sunnyvale, USA). Values of phospho-JNK 1/2 (obtained in three different experiments) were normalized to corresponding total amounts of JNK 1/2 and expressed as percentages of control medium (defined as 100%).

#### *Reverse Transcriptase-Polymerase Chain reaction*

Neutrophils were isolated from human heparinized venous blood from eight healthy volunteers, (age 25–36 years old) after informed consent, by dextran sedimentation followed by centrifugation on Ficoll–Hypaque density gradient, as previously described [12]. Total RNA was extracted from neutrophils with the Nucleospin kit and on-column DNase digested (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. The concentration and purity of RNA were determined by spectrophotometry analysis and cDNA was prepared from 200 ng total RNA using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, USA), as suggested by the manufacturer. As negative controls, reverse transcription experiments were realized by omitting the reverse transcriptase enzyme from the reactions. PCR were performed using primer pairs specific for human

GPR109A and GPR 109B based on the nucleotide sequences available in GenBank (Table 1). The expression of the human ribosomal protein S13 (RPS13) was used as an internal control for the PCR reaction. The PCR products were separated by electrophoresis in a 1% agarose gel and visualized by UV illumination in the presence of SYBR Safe (Invitrogen Corporation, Carlsbad, USA); their molecular identity was confirmed by specific endonuclease digestion.

### *Statistical analysis*

Metabolic syndrome patients were compared to healthy subjects using Pearson's chi-square test or Fisher's exact test, when appropriate, for the comparison of qualitative variables. Continuous variables and *in vitro* results were expressed as mean ( $\pm$  SD). One-way ANOVA was used for multiple group comparison, while Student's *t* test for two group comparison. Spearman's rank correlation coefficients were used to assess correlations between serum-induced neutrophil migration to CCL3 and, respectively, circulating levels of glucose, insulin and NEFA. All analyses were done with GraphPad InStat software (San Diego, CA). Differences were accepted as significant when  $P < 0.05$ .

## **Results**

### *Patient characteristics*

Metabolic syndrome was diagnosed accordingly to the NCEP ATP III criteria. As shown in Table 2, elevated waist circumference was observed in all metabolic syndrome patients. Baseline clinical characteristics and biological parameters in metabolic syndrome patients and healthy subjects are described in Table 3. There was no significant difference between metabolic syndrome patients and healthy controls in terms of age, sex, red blood cells, platelets and cholesterol. As expected, other NCEP ATP III parameters, serum insulin, HOMA index and BMI were different between the two groups [15]. Despite in the

physiological range, total WBC, neutrophil and lymphocyte counts and fibrinogen levels were significantly increased in metabolic syndrome patients as compared to healthy controls.

*Single administration of Acipimox reduces serum levels of insulin and NEFA in metabolic syndrome*

A single dose (500 mg) of Acipimox was orally administered at 8.00 am to metabolic syndrome patients or healthy controls. Just before (time 0) and 2h-5h after Acipimox administration, serum and plasma were collected (Figure 1). At time 0, circulating levels of glucose, insulin and NEFA were significantly increased in metabolic syndrome patients as compared to healthy controls (Figure 2A-C). Serum glucose was not significantly modified after Acipimox assumption in both groups (Figure 2A). Conversely, insulin and NEFA was significantly reduced from time 0 in metabolic syndrome patients (Figure 2B and C). At any time points investigated, NEFA was significantly increased in metabolic syndrome patients as compared to healthy controls (Figure 2C).

*Acipimox administration was associated with the reduction of metabolic syndrome serum-induced neutrophil migration towards the CCL3*

At time 0 and 2h after Acipimox administration, pre-incubation with metabolic syndrome serum induced migration towards CCL3 in human neutrophils isolated from healthy donors (Figure 3A). Pre-incubation with metabolic syndrome serum (collected at 3h-5h after Acipimox administration) did not modify neutrophil locomotion to CCL3. This pro-atherosclerotic effect (mimicking *in vitro* neutrophil recruitment within atherosclerotic plaques) was not observed by incubating cells with healthy control serum (Figure 3A). Pre-incubation with serum from metabolic syndrome or healthy subjects did not induce any effect on neutrophil migration towards CXCL8 (Figure 3B).

*Acipimox inhibits neutrophilic locomotion to CCL3 in metabolic syndrome through its lowering activity on circulating levels of insulin and NEFA*

We recently showed that pre-treatment with high levels of recombinant insulin induced neutrophil migration in response to CCL3 via the activation of JNK 1/2 intracellular pathway. To investigate if the effects of metabolic syndrome serum on neutrophil locomotion was possibly mediated by insulin, we assessed potential correlations between levels of circulating metabolic parameters and serum-induced on migration. At time 0 and 2h after Acipimox administration, strong positive correlations were found between serum-induced neutrophil migration to CCL3 and both insulin and NEFA levels (Table 4). To assess the direct role of insulin, we performed insulin-immunodepletion in sera obtained at time 0 from metabolic syndrome patients. Figure 4A confirmed that pre-treatment with metabolic syndrome serum increased neutrophil migration towards CCL3. Conversely, after insulin immunodepletion, the same sera did not induce any effect on neutrophil migration CCL3 (Figure 4A). Accordingly, insulin immunodepletion also abrogated the associated activation of JNK 1/2 activation (Figure 4B and C). To verify if the beneficial effects on neutrophilic inflammation were possibly associated with the direct activity of Acipimox, we firstly investigated the expression of Acipimox receptor (GPR109) on human primary neutrophils. Figure 5A shows that mRNA of both isoforms (GPR109A [high affinity] and GPR109B [low affinity]) of Acipimox receptor were expressed in human neutrophils. Then, we tested recombinant insulin-induced neutrophil migration, pre-incubating cells in the presence or absence of different concentrations (5-500  $\mu$ M) of Acipimox. No effect was induced by pre-treatment with Acipimox on insulin-mediated neutrophil migration to CCL3 (Figure 5B). These data indicated that Acipimox treatment in metabolic syndrome inhibited neutrophil pro-atherosclerotic functions through the reduction of circulating levels of insulin.

## **Discussion**

In the present article, we demonstrated that a single oral administration of Acipimox (500 mg) was associated with marked improvements of the levels of insulin and NEFA in metabolic syndrome patients. These metabolic effects of Acipimox were previously shown in chronic (weeks) treatment studies enrolling non-insulin dependent diabetes mellitus (NIDDM) [17], or hyperlipoproteinaemic patients [18], or subjects with both diseases concomitant [19]. More recently, Acipimox has been also investigated in acute (few days) treatment studies in normal and diabetic subjects, confirming promising improvements in insulin sensitivity and NEFA levels [20, 21]. In general, chronic treatments of Acipimox used daily doses greater (about 1200 mg) than those tested in short-term studies (250-500 mg). On the basis of these evidences, the present study was designed by administrating a single dose of 500 mg at 8.00 am to fasted metabolic syndrome patients as compared to healthy controls. The acute use of Acipimox in metabolic syndrome patients with fasting hyperinsulinemia and high levels of NEFA represents another novelty of our study. This aspect leaded to us to investigate the potential direct role of insulin and Acipimox on atherosclerotic inflammation associated with metabolic syndrome. This research approach was further supported by the recent discovery of the direct pro-atherosclerotic activities of insulin [22, 23] and the potential pleiotropic activities of Acipimox through the binding with its receptor GPR109 [10, 24, 25]. High levels of recombinant insulin have been previously shown to induce neutrophil recruitment towards the pro-inflammatory CC chemokine CCL3 that is expressed in atherosclerotic plaques [22, 26]. Since neutrophil migration within atherosclerotic plaques increases plaque vulnerability, this effect could be of pathophysiological relevance to reduce acute cardiovascular events [26, 27]. In addition, insulin-induced neutrophil locomotion to CCL3 has been shown as associated with the activation of the intracellular JNK 1/2 pathway [22]. In the present article,

we showed that insulin in sera from metabolic syndrome patients induced neutrophil migration in response to CCL3. This was supported by five independent experiments: 1. insulin levels in metabolic syndrome sera were significantly higher than those detected in healthy subjects; 2. only pre-treatment with metabolic syndrome sera with elevated insulin levels (time 0 and 2 hours after Acipimox administration) induced neutrophil recruitment to CCL3; 3. insulin-immunodepletion in metabolic syndrome sera abrogated serum-neutrophil migration; 4. insulin serum levels positively correlated with serum-induced neutrophil migration. 5. JNK 1/2 activation associated with neutrophil migration was increased in the presence of metabolic syndrome sera and abrogated when incubated with the same serum insulin-immunodepleted. Thus, confirming previous experiments with recombinant insulin [22], these data strongly support the crucial role of insulin in neutrophil-mediated atherosclerotic inflammation. In particular, experiments performed with metabolic syndrome serum immunodepleted of insulin further reduced a potential involvement of other soluble mediators commonly altered in metabolic syndrome, such adipocytokines [6]. On the other hand, we also observed that, in the first hours after Acipimox administration, metabolic syndrome serum lose its potent priming activity on neutrophil locomotion. This effect was probably due to the associated reduction of insulin serum levels. However, a potential direct anti-inflammatory role of Acipimox could not be excluded. To verify this hypothesis, we pre-incubated neutrophils with different concentrations (5-500  $\mu$ M) of Acipimox, previously reported as effective in *in vitro* studies [8]. Despite mRNA GRP109 (Acipimox receptor) expression was shown on naïve human neutrophils, results demonstrated that Acipimox did not modify recombinant insulin-induced migration to CCL3.

In conclusion, we showed that single administration of Acipimox was associated with the reduction of insulin and NEFA levels in metabolic syndrome patients. Decrease in insulin concentrations abrogated *in vitro* neutrophil recruitment towards the pro-inflammatory CC

chemokine CCL3. On the other hand, Acipimox did not directly interfere with insulin-neutrophil migration. These results suggest that Acipimox treatment in metabolic syndrome could be a promising approach to reduce insulin levels and the associated pro-atherosclerotic activities of this hormone.

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### **Declaration of interests**

None declared.

### **Contributor statements**

Fabrizio Montecucco, FD, GLV designed the study, performed experiments, wrote the article. MB, SM, AP, GP, SL performed experiments. AQ, François Mach analyzed and interpreted the data, drafted made the manuscript. All authors performed a critical revision of the manuscript.

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## Legend to figures

### Figure 1. Study design.

Treatment with Acipimox was tested in metabolic syndrome patients (n=11) and healthy controls (n=3). After a standard meal of 600 Kcal at 8.00 pm the day before Acipimox test, subjects were instructed to remain fasted and avoid any physical activity till the end of the test. The day of the test, at 8.00 am, a single dose (500 mg) of Acipimox was orally administrated to metabolic syndrome patients or healthy controls. Just before (time 0) and 2h-5h after Acipimox administration, serum and plasma were collected.

**Figure 2. Single administration of Acipimox reduces circulating levels of insulin and NEFA in metabolic syndrome patients.** Serum levels of glucose (A) and insulin (B) and plasma levels of NEFA (C) were evaluated just before (time 0) and 2h-5h after the administration of 500 mg Acipimox in metabolic syndrome patients (n=11) and healthy subjects (n=3). Data are expressed as mean  $\pm$  SD. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 vs. metabolic syndrome. # P<0.05, ## P<0.01, ### P<0.001 vs. metabolic syndrome at time 0.

**Figure 3. Acipimox administration was associated with the inhibition of metabolic syndrome serum-induced neutrophil migration towards CCL3.** Neutrophils were pre-incubated with serum obtained from metabolic syndrome (n=11) and healthy (n=3) subjects at time 0 or after 2h-5h from Acipimox administration. After washing three times, cell migration towards control medium, 30 nM CCL3 or 10 nM CXCL8 was tested. Data are expressed as mean  $\pm$  SD of net migration of neutrophils obtained from three different healthy donors ( $\mu$ m) towards CCL3 (A) and CXCL8 (B). \*\* P<0.01, \*\*\* P<0.001 vs. metabolic syndrome. ### P<0.001 vs. metabolic syndrome at time 0.

**Figure 4. Insulin immunodepletion of metabolic syndrome sera reduces neutrophil migration towards CCL3 and associated JNK 1/2 phosphorylation.**

**A.** Neutrophils were pre-incubated with normal or insulin-immunodepleted serum obtained from metabolic syndrome (MS, n=11) subjects at time 0 or after 2h from Acipimox administration. After washing three times, cell migration towards control medium or 30 nM CCL3 was tested. Data are expressed as mean  $\pm$  SD of migration of neutrophils obtained from three different healthy donors ( $\mu$ m). \*\*\* P<0.001 vs. MS-induced migration to medium. # P<0.05, ### P<0.001 vs. MS-induced migration to CCL3. **B.** Representative western blot of JNK 1/2 activation in neutrophils stimulated with control medium (for 3 min, negative control), recombinant-insulin (r-Ins, 300 mU/l, for 3 min, positive control), serum from a metabolic syndrome patient collected at time 0 (MS #1, for 3 min), serum from another metabolic syndrome patient collected at time 0 (MS #2, for 3 min), or the same sera immunodepleted of insulin (respectively, i-MS #1 and i-MS #2, for 3 min). **C.** Quantification of densitometries of JNK 1 and JNK 2 activation 3 different western blot experiments. Results obtained in the presence of serum from metabolic syndrome patients (normal [MS] or insulin-immunodepleted [i-MS]) were pooled. Data are expressed as mean  $\pm$  SD. \* P<0.05, \*\*\* P<0.001 vs. medium. ### P<0.001 vs. MS.

**Figure 5. Acipimox does not directly influence insulin-induced neutrophil migration towards CCL3.**

**A.** Representative agarose gel of PCR products from naïve human neutrophils (isolated from healthy donors 1-8) showing mRNA expression of Acipimox receptors GRP109A and GPR109B. **B.** Effect of pre-incubation with different concentrations (5-500  $\mu$ M) of Acipimox on control medium- or 300 mU/l recombinant insulin (r-Ins)-induced neutrophil migration towards 30 nM CCL3. Data are expressed as mean  $\pm$  SD of net migration to CCL3 of

neutrophils obtained from three different healthy donors ( $\mu\text{m}$ ). \*  $P < 0.05$ , \*\*\*  $P < 0.001$  vs. medium-induced migration.