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TESI DI DOTTORATO

"The adipokine apelin-13 induces expression of prothrombotic tissue factor"

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1.INTRODUCTION

1.1 ADIPOCYTES, ADIPOKINES AND ATHEROSCLEROTIC DISEASE

Obesity, a disease in which adipose tissue is largely represented, is actually considered as strong cardiovascular risk factor (1, 2). Thus, although it is established that increase of visceral fat is causally involved in the development of cardiovascular disease, the possible link between these entities is not completely understood yet. Indeed, the current point of view about the adipose tissue is actually changed, indicating that adipocytes have no longer to be considered only storage cells for fat, but they have to be now recognised as cells able to produce and secrete a large variety of active substances named adipokines. Several adipokines have been extensively characterised and are considered of particular interest in cardiovascular pathophysiology (3, 4). Conversely, other novel adipokines, such as apelin, have been poorly investigated. Apelin is expressed as a 77 aminoacid long pre-protein and is cleaved by the cells to produce endogenous peptides of various sizes such as apelin 36, apelin 17, apelin 13 and apelin 12 (5). All these apelin isoforms have the same C-terminal aminoacids and it has been demonstrated that apelin 13 has higher biological activity with respect to apelin 36. Moreover, apelin 12 was found to exert more potent biological activity than apelin 13 while shorter apelin fragment containing the last 10 aminoacids did not show any biological activity (6). These data indicated that the 12 C-terminal residues corresponding to apelin 12 are the minimal structure core able to underline activity of this adipocytokine. Specifically, the 12 C-terminal aminoacids seem to play a pivotal role for apelins binding to their receptor (APJ). Thus, all apelin isoforms, including both apelin 12 and apelin 13, bind to the same receptor on target cells (7). Activation of APJ by apelin results in downstream effects and several evidences indicate that the main target of apelins should be the

cardiovascular system (8). Indeed, the role of apelin in cardiovascular system physiology has been studied in regard to vessel vasodilation (9) and myocardial contraction (10). Moreover, it has been recently demonstrated that this adipokine is able to induce expression of adhesion molecules in endothelial cells in vitro, suggesting that it might play an important role for the initiation and progression of the atherosclerotic disease (11). Increasing evidences have assigned a central role to thrombosis in the progression and complication of atherosclerosis and have linked it to the clinical occurrence of acute coronary syndromes (ACS) (12–14).

1.2 TISSUE FACTOR AND PATHOPHYSIOLOGY OF ACUTE CORONARY SYNDROMES (ACS)

Several studies have clearly indicated that tissue factor (TF), the key initiator of extrinsic coagulation pathway, plays a pivotal role in the pathophysiology of ACS by triggering the formation of intracoronary thrombi following endothelial injury (15–17). Cells that are in direct contact with blood and are a potential source of TF include endothelial cells and monocytes (18). Aberrant TF expression by these cells is thought to be responsible for the thrombus formation observed in several disease such as cancer, septic shock, and, as reported above, in ACS (19). In the present study, we provide support, in vitro, for the hypothesis that apelin might effectively play an active role in the pathophysiology of coronary acute events by promoting a prothrombotic state in endothelial cells and monocytes through induction of TF.

2. METHODS

2.1 HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Basel, Switzerland) were grown in EGM 2 medium with 10 % FBS and used at passages 2 to 5. To investigate the apelin effects on TF expression and activity, cells were enzimatically harvested and counted in a haemocytometer and sub cultured in 24-well plates at an initial density of about 5 × 104 cell/well, while at confluence, cell density was of about 8.5 × 104 cell/well. For other set of experiments, cells were grown in 100 mm cell plates and, at confluence, cell density was of about 2 × 106 cells per plate. At confluence, cell were starved in serum-free medium for 24 hours (h) and then used in the different set of experiments.

2.2 HUMAN MONOCYTE LINE CELLS

THP-1, human monocyte line cells, were cultured in serum media (RPMI 1640 supplemented with 2.5 g/l D (+) glucose, 1 mM Sodium Pyruvate, 10 mM HEPES, 1 % Penicillin/ streptomycin, and 10 % heat-inactivated fetal calf serum) at 37°C in 5 % CO2. Cells between passage 3 and 10 were used for the experiments. THP-1 cells were plated at the density of 1.0–1.5 × 106 /ml in sixwell plates. At confluence, cell were starved in serum-free medium for 24 h and then used in the different set of experiments.

2.3 APELIN 12 AND 13

Apelin 12 and Apelin 13 (Sigma Chemical Co., St Louis, MO, USA) used in all studies were analysed founding endotoxin level to be < 0.125 EU/ml (< 12.5 pg/ml) by Limulus assay (Bio Whittaker, Walkersville, MD, USA). All media, and water were also tested and endotoxin level found to be < 0.125 EU/ml. In addition, in order to avoid a possible bias from contaminating LPS, all experiments were then repeated in the presence of Polymyxin B (100 µg/ml).

2.4 EFFECTS OF APELINS ON TF SURFACE EXPRESSION AND ACTIVITY IN ENDOTHELIAL CELLS

HUVECs cultivated as above were washed with phosphate-buffered saline (PBS) and then incubated with increasing concentrations of apelin 12 and apelin 13 (10–9, 10–8, 10–7 M) for 6 h. TF expression in cell lysates was determined with Western blotting. The samples (30 µg) were treated with SDS-PAGE sample buffer, followed by heating and then subjected to 10 % gel. The protein was transferred onto membranes with a semidry transfer unit. TF was detected with a specific antibody (1: 100, American Diagnostica Inc, Greenwich, CT, USA). All experiments were performed in triplicate. TF expression on cell surface was investigated with FACS analysis. After incubation, endothelial cells were detached with 10 mmol/l EDTA in PBS (without trypsin) and stained with FITC-labelled monoclonal antibody (Pharmingen, Franklin Lakes, NJ, USA) against TF, or with the appropriate isotype IgG (phycoerythrin or FITC) as control. Fluorescence intensity of 9,000 cells for each sample was quantified by a FACSCalibur analyzer (BectonDickinson, Franklin Lakes, NJ, USA). To evaluate whether apelin-induced TF was functionally active, TF procoagulant activity was

determined by a two-step colourimetric assay, based on the ability of TF to promote generation of coagulation factor (F)Xa, as previously described (20). Briefly, after stimulation with apelin 12 or apelin 13, cells were incubated with 1 nM of recombinant human FVIIa (Novo Nordisk A/S Gentofte, Denmark), followed by 100 nM of purified human factor X (Calbiochem-Novobiochem, La Jolla, CA, USA) and 5 mM CaCl2 for 15 minutes (min) at 37 °C. A chromogenic substrate, specific for factor X (Cromozym X, Roche Diagnostics, Mannheim, Germany, 0.5 mmol/l) was then added and incubated for 30 min at 37 °C. The reaction was stopped by adding 200 µl/ml of sample of a 30 % solution of acetic acid and the change in optical density at 405 nm was quantified with a spectrophotometer. Purified factor Xa of known concentration (Sigma Chemical Co.) allowed generation of calibration curves. To evaluate whether apelin-induced expression of TF resulted from de novo synthesis, in another set of experiments, HUVECs were pre-incubated for 120 min with cycloheximide (10 µg/ml), an inhibitor of protein synthesis, or with 5, 6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB, 10 µg/ml), an inhibitor of DNA transcription, before adding apelin. In additional control experiments cells were pre-incubated for 10 min with an antibody against human apelin receptor (Abcam Inc, Cambridge, MA, USA) or with a mouse monoclonal antibody against human TF (5 µg/ml, American Diagnostica Inc). Positive control experiments included cells incubated for 6 h with lipopolysaccharide (LPS) (50 µg/ml). Six different experiments were performer for each experimental condition. Since it has been demonstrated that effects of apelin on its receptor might be mediated by Gi alpha subunits (21), in an additional set of experiments we have investigated whether Pertussis Toxin (PTX, Sigma Chemical Co.) a substance able to uncouple Gi/Go proteins, was able to abrogate the effects of this adipokine on TF, in HUVECs.

Thus, cells were stimulated with PTX (25 ng/ ml for 16 h), and then TF expression was determined as described above.

2.5 EFFECTS OF APELINS ON TF-mRNA LEVELS IN ENDOTHELIAL CELLS

The effect of apelin 12 and apelin 13 on TF-mRNA was investigated by real-time reverse transcription (RT) analysis as previously described (20). Briefly, HUVECs were incubated with apelin 12 and apelin 13 (10–7 M, concentration chosen on the basis of FACS analysis) for 30 min. Then, cells were washed with PBS and then fresh medium (EGM 2 containing 0.1 % serum) was added. Total mRNA was extracted by cell cultures using TRIzol reagent (GIBCO, Carlsbad, CA, USA), at baseline and 60 min after apelin stimulation and TF mRNA levels were examined by real-time RT and polymerase chain reaction (PCR) by LightCycler (Roche Diagnostics, Basel, Switzerland). In positive control experiments, HUVECs were incubated with LPS (50 µg/ml, Sigma Chemical Co.), for 30 min and then mRNA was extracted at 60 min. Total mRNA was extracted from cell cultures using TRIzol reagent (GIBCO), according to the manufacturer's instructions. RT was performed using mMLV (GIBCO) and 100 ng of the RNA samples from each culture condition. Samples were run in triplicate in 50 µl reactions by using an ABI PRISM 5700 sequence detector system (Applied Biosystems, Foster City, CA, USA). Samples were incubated at 50 °C for 2 min, 95 °C for 10 min and then underwent 40 cycles at 95 °C for 15 seconds (s) and 60 °C for 1 min. Specific oligonucleotides for human GAPDH and human TF were designed on the basis of published sequences using PRIMER EXPRESS Software (Applied Biosystems) and validated for their specificity.

SYBR-green chemistry was used to detect fluorescence and an internal standard (Applied Biosystems) was used for quantization of the message. The results were analysed using a comparative method, and the values were normalised to the GAPDH expression and converted into percentage change. Three different experiments were performed for each experimental condition.

2.6 EFFECTS OF APELINS ON NUCLEAR FACTOR NF-kB ACTIVATION IN ENDOTHELIAL CELLS

To investigate whether expression of TF induced by apelin involved activation of the NF-kB pathway, we performed luciferase assays. HUVECs cells (3×105) were transfected with 1.5 μg of the Ig-kB-luciferase reporter gene plasmid, containing NF-kB-binding sites of immunoglobulin promoter region, and, 24 h after transfection, were treated with apelin 12 or apelin 13 (10–7 M). After 3 h of incubation at 37 °C, cell extracts were prepared and reporter gene activity was determined by the luciferase system (Promega, Madison, WI, USA). A pRSV-β-galactosidase vector (0.5 μg) was used to normalise for transfection efficiencies.

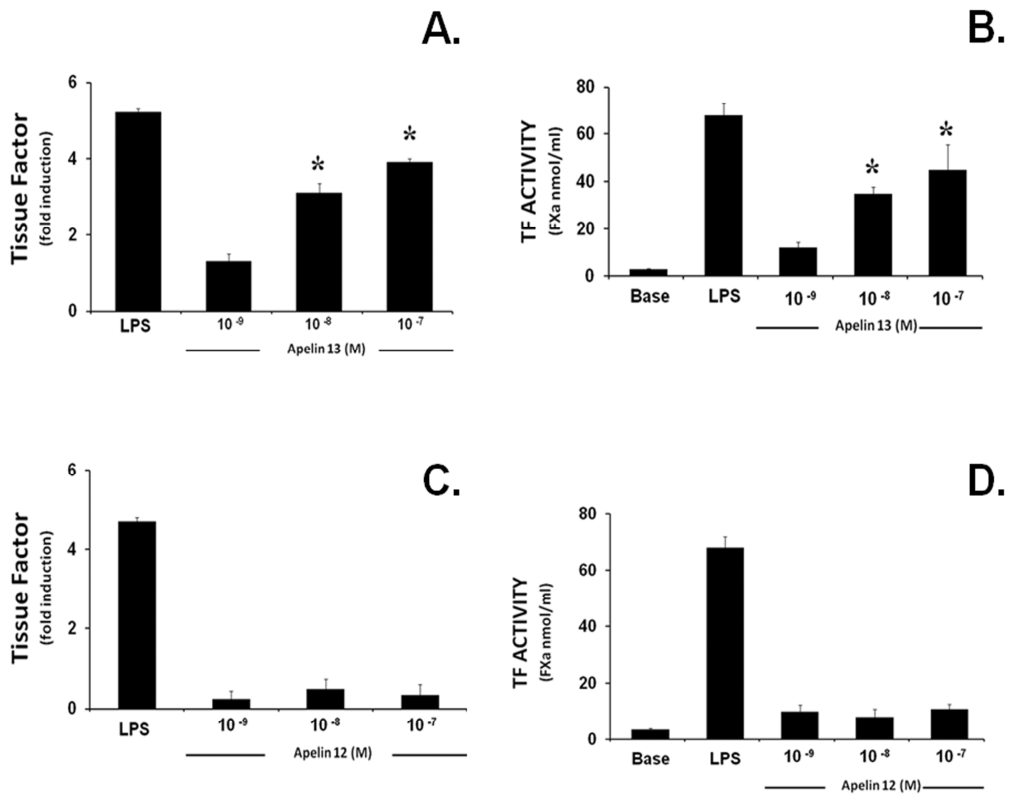


***Figure 1**: Effects of apelin 12 and apelin 13 on TF expression/activity in HUVECs.*
***A and C)** Dose-response effects of apelin 13 and apelin 12 on TF expression in HUVECs determined by FACS analysis. Apelin 13 caused progressive increase of TF expression, in a dose-dependent fashion. On the contrary, increasing concentrations of apelin 12 did not cause any significant effect on TF expression.*
***B and D)** Effects of apelin 13 and apelin 12 on TF activity in HUVECs, determined by a two-step colourimetric assay based on the ability of TF/FVIIa to promote generation of coagulation FXa. TF activity reflects results observed for TF expression, confirming that TF was functionally active. Each bar represents the mean ± SD of six different experiments. LPS represents positive control experiments. * = p < 0.005 vs unstimulated, control cells.*

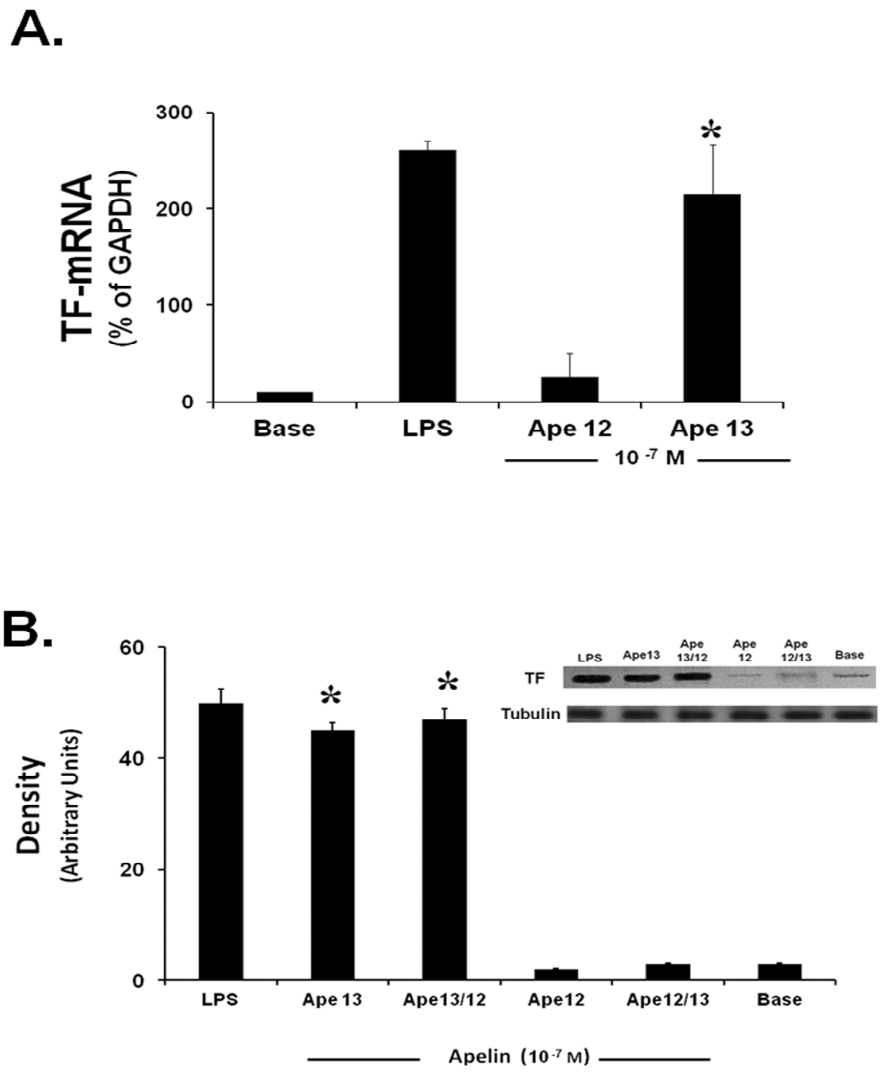


***Figure 2**: Effects of apelin 12 and apelin 13 on TF mRNA and TF protein in HUVECs.*
A) Effects of apelin 13 and apelin 12 on TF-mRNA levels in HUVECs assessed by real-time quantitative PCR. TF mRNA was undetectable at baseline (Base) in unstimulated cells. Apelin 13 caused increase in TF mRNA levels at 60 min, as compared to unstimulated cells. On the contrary, apelin 12 did not stimulate TF-mRNA transcription. LPS (50 µg/ml) represents positive control. Each bar represents the mean ± SD of three different experiments. Data are expressed as % change vs control gene represented by GAPDH. (* = p< 0.005 vs Base).
B) TF expression evaluated by Western Blot analysis in HUVEC incubated with apelin 12, apelin 13, or alternatively pre-incubated with apelin 12 and apelin 13 in combination. Tubulin served as loading control. Graphs are summary of data from three separate experiments (mean ± SD; * = p< 0.005 vs Base); Insert shows result of a representative experiment.

2.7 EFFECTS OF APELINS ON TF SURFACE EXPRESSION AND ACTIVITY IN MONOCYTES

THP-1 cultivated as above were washed with PBS and incubated with increasing concentrations of apelin 12 and apelin 13 (10–9, 10–8, 10–7 M) for 6 h. TF expression on cell surface was investigated with FACS analysis as described above. To evaluate whether apelin-

induced TF was functionally active, TF procoagulant activity was determined by a two-step colorimetric assay, based on the ability of TF to promote generation of coagulation FXa, as above.

2.8 EFFECTS OF APELINS ON TF-mRNA LEVELS IN MONOCYTES

The effect of apelin 12 and apelin 13 on TF-mRNA was investigated by real-time RT analysis. Briefly, THP-1 were incubated with apelin 12 and apelin 13 (10–7 M, concentration chosen on the basis of FACS analysis) for 30 min. Then, cells were washed with PBS and then fresh medium (EGM 2 containing 0,1 % serum) was added. Total mRNA was extracted by cell cultures using TRIzol reagent (GIBCO), at baseline and 60 min after apelin stimulation and TF mRNA levels were examined by real-time RT and polymerase chain reaction (PCR) by LightCycler (Roche Diagnostics) as above. The results were analysed using a comparative method, and the values were normalized to the TBP expression and converted into percentage change. Three different experiments were performed for each experimental condition.

2.9 STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD). Differences between groups were determined by a one-way ANOVA followed by a Student's t test with Bonferroni's correction. A p value < 0.05 was considered statistically significant. In all statistical analysis SPSS 17.0 Statistical Package Program for Windows (SPSS Inc., Chicago, IL, USA) was used.

3. RESULTS

3.1 EFFECTS OF APELINS ON TF SURFACE EXPRESSION AND ACTIVITY IN ENDOTHELIAL CELLS

HUVECs were incubated with increasing concentrations of apelin 12 or apelin 13 (10–9, 10–8, 10–7 M) for 6 h, and then processed to evaluate TF protein levels with Western Blot analysis and TF expression on cell surface with FACS analysis. Western blot showed that TF protein levels increased after stimulation with apelin 13 as compared to baseline TF levels. On the contrary, apelin 12 did not cause any increase of TF. Similarly, TF protein expression did not increase in HUVEC pre-incubated with apelin 12 before adding apelin 13 (Figure 2 B). TF expression on cell surface with FACS analysis was almost undetectable on unstimulated HUVECs, at baseline. Apelin 13 caused progressive increase of TF expression, in a dose-dependent fashion (Figure 1 A). Similarly, TF procoagulant activity, determined by a two-step colourimetric assay, based on the ability of TF to promote generation of coagulation FXa, was almost undetectable at baseline, and progressively increased after stimulation with increasing apelin 13 doses (Figure 1 B). On the contrary, increasing concentrations of apelin 12 did not cause any significant effect on TF expression as well as activity (Figure 1 C and D). Interestingly, TF expression/activity did not increse in cells pre-incubated with apelin 12 before adding apelin 13 (_ Figure 3 A and B). In experiments repeated in the presence of Polymyxin B, apelins had similar effects on TF expression and activity (data not shown). Pre-incubation with cycloheximide (10 μg/ml), an inhibitor of protein synthesis, or with DRB (10 μg/ml), an inhibitor of DNA transcription, significantly inhibited TF expression as well as TF procoagulant activity in cells stimulated with apelin 13 (Figure4 A), suggesting that this adipocytokine is able to induce *de novo* synthesis of TF and these new TF molecules are then expressed in an active form on the cell surface. Control

experiments, performed by pre-incubating cells with a mouse monoclonal antibody directed against human TF, confirmed that only TF molecules have been detected by FACS analysis, and that procoagulant activity measured was really due to TF expression on cell surface after apelin 13 induction (Figure 4 A). Similarly, pre-incubation with an antibody directed against apelin receptor (APJ), abrogated apelin 13 effects on TF expression, witnessing that this phenomenon was dependent by apelin stimulation (Figure 4 A). Interestingly, in an additional set of experiments performed by pre-stimulating cells with Pertussis Toxin (PTX), apelin 13 did not induce TF expression, suggesting that Gi/Go proteins are involved in apelindependent TF expression (Figure 4 A).

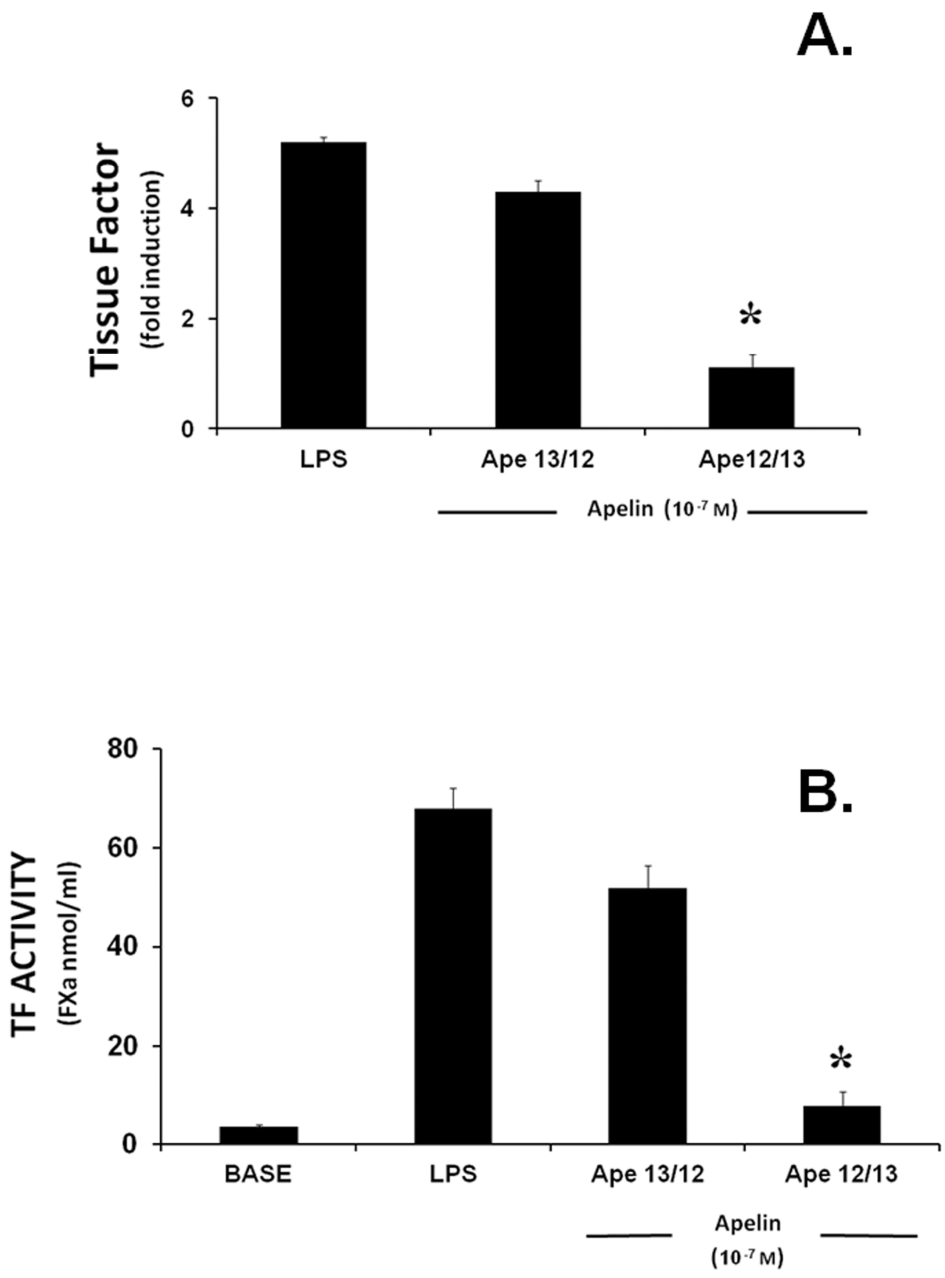


***Figure 3**: TF expression (A) and activity (B) in HUVEC alternatively pre-incubated with apelin 12 and apelin 13 in combination. Expression as well as activity did not increase in cells pre-incubated with apelin 12 before adding apelin 13. Each bar represents the mean ± SD of six different experiments. * = p < 0.005 vs Ape 13/12.*

3.2 EFFECTS OF APELINS ON TF mRNA LEVELS IN ENDOTHELIAL CELLS

Apelins effects of TF-mRNA transcription were investigated by real-time RT analysis. TF mRNA levels were very low in unstimulated HUVECs. Apelin 13 (10–7 M), caused increase in TF Mrna levels at 60 min, as compared to unstimulated cells, as expected (22, 23). On the contrary, apelin 12 did not stimulate TF-mRNA transcription (Figure 2 A). In experiments repeated in the presence of Polymyxin B, Apelins had similar effects on mRNA transcription (data not shown)**.**

3.3 EFFECTS OF APELINS ON NF-kB ACTIVATION

Since we have previously demonstrated that different stimuli are able to up-regulate TF expression *via* NF-kB (22, 23) and given the property of apelin to induce gene target expression through NF-kB activation (11), we investigated whether apelin-mediated TF upregulation was determined by NF-kB activation. Thus, we analysed NF-kB activity in HUVEC cells transfected with a plasmid containing a luciferase reporter gene downstream the promoter region of immunoglobulin k light chain responsive to NF-kB activity and incubated them with apelin 12 or apelin 13. Apelin 13 but not apelin 12 induced NF-kB activation, indicating that this adipocytokine up-regulates TF expression in HUVEC cells *via* NF-kB activation (Figure 4 B). Interestingly, PTX, a substance able to uncouple Gi/Go, caused the significant reduction of NF-kB activity, expressed as luciferase activation (Figure 4 B).

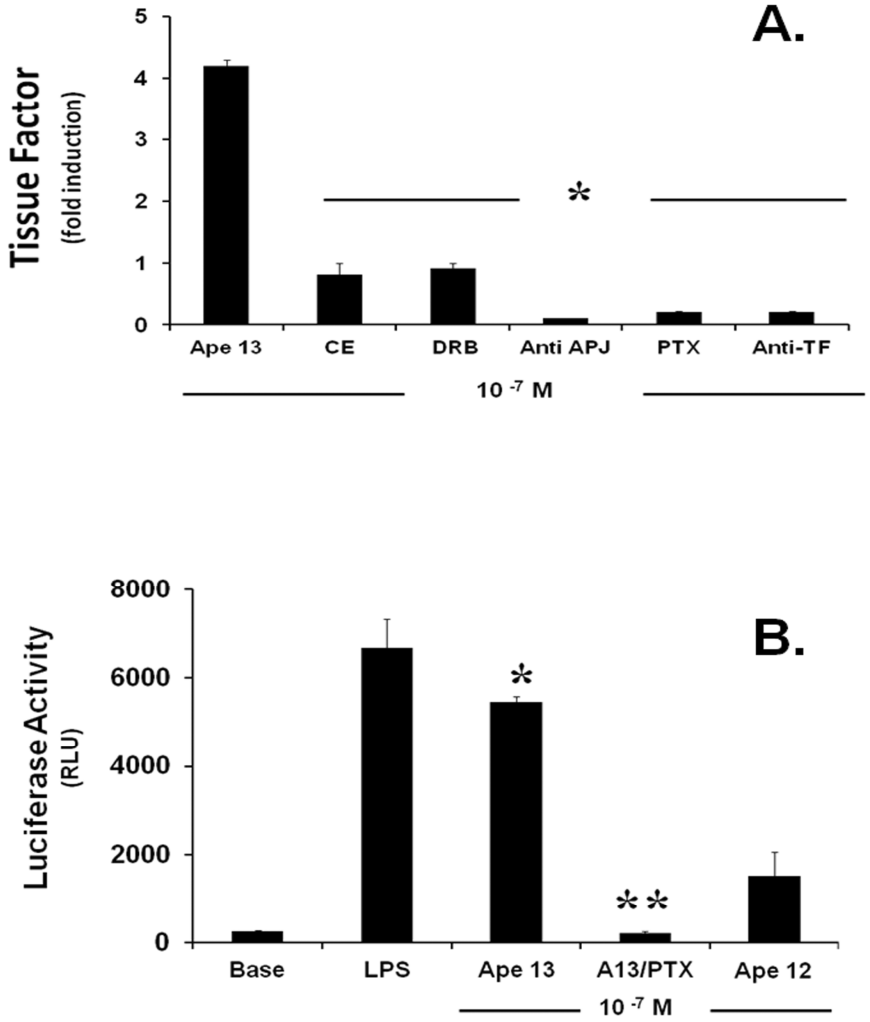


***Figure 4**: TF activity and NF-kB activation in HUVECs. A) Control experiments performed by pre-incubating endothelial cells with cycloheximide (CE), DRB, with a mouse monoclonal antibody directed against human TF (Anti-TF), with an antibody directed against apelin receptor (anti-APJ) and with PTX, a substance able to uncouple Gi/Go proteins. Each bar represents the mean ± SD of six different experiments. * = p < 0.005 vs Ape 13 stimulated cells.*

*B) Apelin 13 mediates TF up-regulation through NF-_B activation. HUVECs were transfected with the Ig-_B-luciferase reporter gene plasmid, containing NF-kB-binding sites of immunoglobulin promoter region, and, 24 h after transfection, were treated with apelin 12 or apelin 13. Results are the means (± SD) of at least three independent experiments, normalised for beta-galactosidase activity of a co-transfected Rous sarcoma virus beta-galactosidase plasmid. * = p < 0.005 vs unstimulated, control cells (Base). PTX, a substance able to uncouple Gi/Go, caused the significant reduction of NF-_B activity. ** = p < 0.005 vs Apelin-13 stimulated cells (Ape 13).*

3.4 EFFECTS OF APELINS ON TF SURFACE EXPRESSION AND ACTIVITY IN MONOCYTES

THP-1 were incubated with increasing concentrations of apelin 12 or apelin 13 (10–9, 10–8, 10–7 M) for 6 h, and then processed to evaluate TF expression on cell surface with FACS analysis. TF expression was almost undetectable at baseline. Apelin 13 caused progressive increase of TF expression, in a dose-dependent fashion (Figure 5 A). Similarly, TF procoagulant activity, determined by a two-step colourimetric assay, based on the ability of TF to promote generation of coagulation FXa, was almost undetectable at baseline, and progressively increased after stimulation with increasing apelin 13 doses (Figure 5 B). On the contrary, increasing concentrations of apelin 12 did not cause any significant effect on TF expression as well as activity (Figure 5 A and B). Interestingly, TF expression/activity did not increse in cells pre-incubated with apelin 12 before adding apelin 13 (Figure 6 A and B). In experiments repeated in the presence of Polymyxin B, apelins had similar effects on TF expression and activity (data not shown).

3.5 EFFECTS OF APELINS ON TF-mRNA LEVELS IN MONOCYTES

The effect of apelin 12 and apelin 13 on TF-mRNA was investigated by real-time RT analysis. TF mRNA levels were very low in unstimulated THP-1. Apelin 13 (10–7 M), caused increase in TF mRNA levels at 60 min, as compared to unstimulated cells. On the contrary, apelin 12 did not stimulate TF-mRNA transcription (Figure 5 C). In experiments repeated in the presence of Polymyxin B, Apelins had similar effects on mRNA transcription (data not shown).

4. DISCUSSION

4.1 DISCUSSION

Several epidemiological studies have clearly demonstrated that human obesity, a disease in which adipose tissue is largely represented, is a strong cardiovascular risk factor causally involved in the development of cardiovascular disease (1, 24). Starting from these epidemiological observations, many efforts have been recently done to investigate the adipose tissue and its relationship with cardiovascular disease. Emerging evidence has transformed the current point of view about this tissue, indicating that adipocytes should no longer be considered only for their role in fat storage, as they are being recognized as cells able to secrete cytokines (25). Specifically, several reports have clearly indicated that some of these adipose-derived cytokines, known as adipokines, exert pro-atherothrombotic effects on vascular cells (22, 23, 26). However, the hypothetical role of other adipokines in the pathophysiology of cardiovascular disease is not completely known yet (4). Apelin is one of the most recently identified adipokines. Indeed, apelin belongs to the family of apelin peptides“ derived by a single gene that exert their effects by binding to a specific receptor (APJ), widely expressed in several tissues including the heart and the vasculature (5, 27). Interestingly, APJ has been detected in cells involved in atherothrombosis such as HUVECs and monocytes/macropaghes (27, 28). Some of these „apelin peptides“ and specifically apelin 12, apelin 13 and 17 have been shown to be involved in several cardiovascular functions but they are still poorly characterized in the pathophisiology of ACS (27). Very few clinical reports have investigated the relationship existing between apelin and ACS (29, 30). It has been demonstrated that plasma levels of this adipokine in patients with ACS were significantly lower than those measurable in control patients, thus excluding that apelin might be

involved in ACS pathophisiology. Indeed, in those reports, the authors have measured plasma concentrations of apelin 12, and not of apelin 13. This is a very important issue because experimental reports about this research issue have investigated the potential role of apelin 13. Specifically, apelin 13 seems invoved in plaque formation and progression because it induces expression of adhesion molecules and stimulates vascular smooth muscle cell proliferation and migration into the neointima (11, 31–33). Moreover, it has been demonstrated that apelin is up-regulated in human atherosclerotic coronary artery where, in the plaques, it co-localises with smooth muscle cells and immune cells, such as monocytes/macrophages, usually involved in the pathophysiology of acute coronary events as well (31). Results of the present study suggest that apelin might be responsible for plaque thrombosis as well. We have demonstrated, using a cell culture model, that apelin 13 induces TF expression in a dose-dependent fashion in cells that are in direct contact with blood and are a potential source of TF, such as endothelial cells and monocytes, which are considered to be responsible for the thrombus formation in ACS (19). Moreover, in HUVEC, we have observed that this phenomenon appears to be mainly related to the synthesis of new TF molecules, since cycloheximide and DRB, an inhibitor of protein synthesis and mRNA transcription, respectively, completely inhibited the apelin effects on TF expression. Of particular pathophysiological interest was the finding that these newly formed TF molecules were functionally active, as demonstrated by the parallel increase in TF-procoagulant activity, which was detectable on the surface of stimulated cells. This observation might have important pathophysiological consequences, considering that endothelial cells are at the interface between the vessel wall and circulating blood coagulation factors; thus, apelin seems to be able to induce a “procoagulant” phenotype in endothelial cells.

Another interesting finding of the present manuscript is that TF expression in HUVEC and monocytes is triggered by apelin 13 and not by apelin 12. Moreover, apelin 13 did not induce TF expression/ activity in cells preincubated with apelin 12, suggesting that this shorter apelin isoform might exert a protective effect on apelin 13 induced TF expression/activity. A possible mechanism by which apelin 12 exerts this protective effect is that the isoforms of apelin differ in receptor binding affinity (34). It is also possible that different ligand isoforms may induce differential receptor trafficking and signalling (7, 35). In fact, it has been postulated that the C-terminal sequence of apelin (conserved in all different apelin isoforms) in involved in receptor binding, and that the N-terminal sequence (different in apelin isoforms), might modulate its interaction with the receptor regulating its internalisation in the cells (36). These hypothetical protective role for apelin 12 is strengthen by recent data indicating that injection of apelin 12 reduces infarct size in rats [37], a pathophysiological phenomenon in which TF-mediated activation of the extrinsic coagulation pathway makes an important contribution (38). Interestingly, we have demonstrated that apelin-induced TF expression was mediated by the G-protein-transcription factor NF-_B axis. Luciferase reporter gene downstream the promoter region responsive to NF-_B was expressed after apelin stimulation and TF expression/activity was abolished by pre-treatment of cells with PTX, which blockades the G-protein system. NF-_B is present but inactive in the cytoplasm of many cells such as lymphocytes, monocytes, endothelial and smooth muscle cells. It seems to be activated by several stimuli during the atherosclerotic process and it is responsible of the expression of inflammatory proteins that actively participate in this process, leading to plaque disruption and acute coronary events (39). Specifically, binding sites for this transcription factor are contained in the promoter for TF (40).

Moreover, NF-_B has been demonstrated to be activated in the unstable plaques of patients with ACS (41, 42).

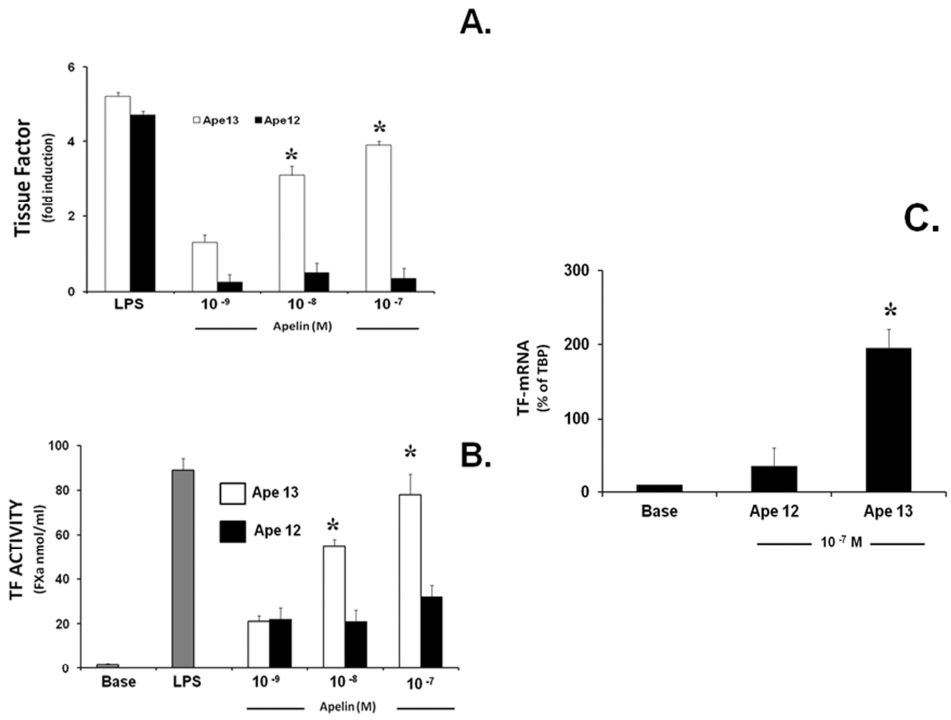
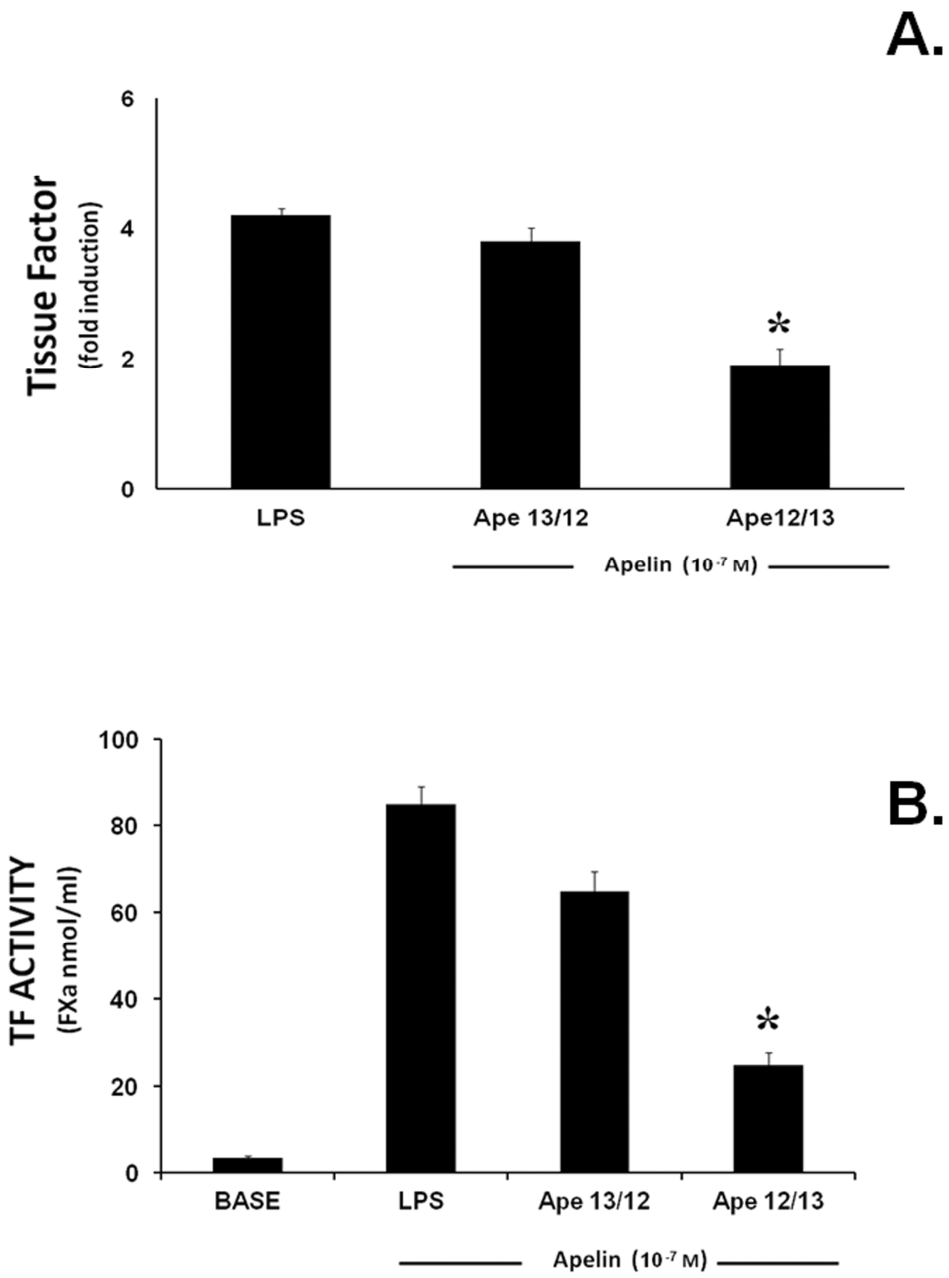


***Figure 5**: Effects of apelin 12 and apelin 13 on TF activity/expression and mRNA in monocytes. **A and B)** TF expression and activity in monocytes stimulated with increasing doses of apelin 12 or apelin 13. Each bar represents the mean ± SD of six different experiments. * = p < 0.005 vs unstimulated, control cells (Base).*
***C)** Effects of apelin 13 and apelin 12 on TF-mRNA levels in monocytes assessed by real-time quantitative PCR. TF mRNA was undetectable at baseline (Base) in unstimulated cells. Apelin 13 caused increase in TF mRNA levels at 60 min, as compared to unstimulated cells. On the contrary, apelin 12 did not stimulate TF-mRNA transcription. LPS (50 µg/ml) represents positive control. Each bar represents the mean ± SD of three different experiments. Data are expressed as % change vs control gene represented by TBP. (* = p< 0.005 vs Base).*



<u>Figure 6</u>: TF activity (A) and expression (B) in monocytes alternatively pre-incubated with apelin 12 and apelin 13 in combination. <u>A and B)</u> TF expression and activity in monocytes alternatively pre-incubated with apelin 12 and apelin 13 in combination. Expression as well as activity did not increase in cells preincubated with apelin 12 before adding apelin 13. Each bar represents the mean ± SD of six different experiments. * = p < 0.005 vs Ape 13/12.

4.2 POTENTIAL LIMITATIONS OF THE PRESENT STUDY

The plasma concentrations of apelin seems to be quite low, because they show disparity from a few picograms per milliliters to several nanograms per milliliter. It has been suggested that this disparity reflects individual variations (43). In line with these observations, several reports are unanimous in indicating that, in ACS patients, levels of apelin 12 are low, but, at the same time, they differ in measurable plasma apelin concentrations. Moreover, all those clinical studies have investigated apelin plasma levels comparing them to plasma levels measured in small control groups and not in the whole population. Finally, clinical studies have measured apelin 12 and not apelin 13. Thus, at this time, it seems impossible to accurately determine the physiological plasma concentrations of this adipocitokine. In the present report, to be in line with experimental literature about apelins, we have used apelin in a range of concentrations that has been previously used to evaluate how this adipocytokine affects CAMs expression in HUVECs (11). However, it should be kept in mind that plasma apelin concentrations might only loosely reflect its tissue levels and that locally, that is, within the arterial wall, apelin might be present in amounts sufficiently high to exert relevant cellular effects. Indeed, apelin is highly expressed in several tissues including cells normally represented in the nornal and atherosclerotic vessel wall such as smooth muscle, endothelial cells or monocytes/macrophages. In conclusion, in previous studies we have investigated the role of some adipocitokynes in the pathophysiology of ACS indicating their potential involvement in these phenomena (22, 23, 26). The present study points out that apelin(s) are other adipocytokine potentially involved in the athero-thombotic disease, shedding for the first time a newer light on the different role played by

two different isoforms in modulating this phenomenon. Of note, we have demonstrated that apelin 13 and not apelin 12 is able to trigger TF expression in cells that are in direct contact with blood and are a potential source of TF, such as endothelial cells and monocytes that have a key role in the pathophysiology of acute coronary events. Moreover, we indicate that apelin 12 exerts a protective effect on apelin 13 induced TF expression/activity. These results, taken together with those obtained in patients with ACS in whom low apelin 12 levels were measured, permit to speculate that apelin 12 might exert a "protective" effect against the "pro-atherothrombotic" apelin 13. Further studies are warranted to clarify whether these mechanisms are also important in the clinical setting.

5.REFERENCES

5.1 REFERENCES

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