UNACYLATED GHRELIN RESCUES ENDOTHELIAL PROGENITOR CELL FUNCTION IN INDIVIDUALS WITH TYPE 2 DIABETES

Running title: UAG improves vascular repair

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**Objective:** Acylated ghrelin (AG) is a diabetogenic and orexigenic gastric polypeptide. These properties are not shared by the most abundant circulating form, which is unacylated (UAG). An altered UAG/AG profile together with an impairment of circulating endothelial progenitor cell (EPC) bioavailability were found in diabetes. Based on previous evidence for the beneficial cardiovascular effects of AG and UAG, we investigated their potential to revert diabetes-associated defects.

**Research Design and Methods:** Healthy human subjects, individuals with type 2 diabetes (T2D) and ob/ob mice were AG- or UAG-infused. EPC mobilization in patients and mice was evaluated and the underlying molecular mechanisms investigated in bone-marrow (BM) stromal cells. Recovered EPCs were also evaluated for the activity of senescence regulatory pathways and for NADPH oxidase activation by knocking-down p47\text{phox} and Rac1. Finally, UAG modulation of human EPC vasculogenic potential was investigated in an *in vivo* mouse model.

**Results:** Neither AG nor UAG had any effect in healthy subjects. However, systemic administration of UAG, but not AG, prevented diabetes-induced EPC damage by modulating the NADPH oxidase regulatory protein Rac1 and improved their vasculogenic potential both in individuals with T2D and in ob/ob mice. Additionally, unlike AG, UAG facilitated the recovery of BM-EPC mobilization. Crucial to EPC mobilization by UAG was the rescue of nitric oxide synthase (eNOS) phosphorylation by Akt, as UAG treatment was ineffective in eNOS knockout mice. Consistently, EPCs expressed specific UAG binding sites, not recognized by AG.

**Conclusions:** These data provide the rationale for clinical applications of UAG in pathological settings where AG fails.
Ghrelin is a 28 amino-acid peptide that circulates in both acylated (AG) and, more abundantly, unacylated forms (UAG) (1). Historically, AG, usually referred as ghrelin, has been considered the only active form of the peptide. It recognizes the Gq-coupled growth hormone (GH) secretagogue receptor type 1a, denoted as GHS-R1a (2) mediating its GH-releasing properties as well as other significant neuroendocrine actions (3). In contrast, the unacylated form (UAG) does not bind the GHS-R1a and is devoid of GH-secretagogue activity (3). Nevertheless, both AG and UAG share affinity for common binding sites which mediate vascular activities in terms of vasodilation, inhibition of cardiomyocyte and endothelial cell apoptosis (3). Furthermore, UAG effects, different from those elicited by AG, have also been demonstrated (4), suggesting the existence of an additional unidentified receptor for UAG.

Besides expression in several tissues (5), the cardiovascular system included (6; 7), ghrelin is mostly produced by the stomach. In particular, in humans AG reduces insulin sensitivity and exerts orexigenic activity, while UAG has opposite effects (8). Circulating total ghrelin levels are negatively associated with body mass index (9) and ghrelin secretion is reduced in obese (10) and in individuals with T2D (11), possibly as a compensatory mechanism protecting against hyperglycaemia. Interestingly, a relative excess of AG compared to UAG has been reported in clinical conditions marked by insulin resistance (12), raising the possibility that the altered UAG/AG ratio could play a role in the altered glucose metabolism and on its ongoing complications.

Among such complications, accelerated vascular disease is widely recognized as the major cause of disability and death in individuals with T2D. Endothelial injury is thought to represent a crucial step in the initiation and progression of atherosclerotic vascular disease in this setting (13). Previous data support the central role of advanced glycated end products (AGE) (14) and of NADPH oxidase-mediated reactive oxygen species (ROS) production in impaired vascular remodelling associated with diabetes (15). NADPH oxidase (Nox) consists of a membrane bound catalytic subunit and several cytosolic regulatory subunits (p47phox and p67phox). Moreover, the GTPase-bound Rac1 is required for the functional assembly of the holoenzyme (15).

Vascular remodelling relies on resident endothelial cells and on circulating endothelial progenitor cells (EPCs): early circulating angiogenic cells (CACs) and late EPCs (16;17). Although they share several common features, they have distinct features with respect to morphology, proliferative potential, and functional characteristics (18). Compelling evidence indicates that changes in their number and functional activities are closely associated with cardiovascular risk factor profiles (19; 20), impacting on their delivery to sites of ischemia where angiogenesis might be required. Indeed, treatment with certain cytokines to induce bone-marrow (BM) mobilization of EPCs has been shown to be cardioprotective (21). EPC mobilization strictly depends on local secretion and activation of the matrix metalloproteinase 9 (MMP9) in the haematopoietic and stromal compartments of the BM (22). In turn, activated MMP9 converts the membrane-bound form of the Kit ligand (mbKitL) into a soluble form (sKitL) that promotes haematopoietic and endothelial progenitor cell proliferation and facilitates their mobilization into the circulation (22). Since an impairment of progenitor cell mobilization has also been reported in mice lacking endothelial nitric oxide synthase (eNOS) (23), eNOS may also be involved in
the control of progenitor cell delivery to sites of neovascularization. Consistently, pathological settings, characterized by reduced systemic NO bioavailability, also show defective EPC mobilization and compromised vascular regenerative processes (24; 25).

Based on previous evidence for cardiovascular protective effects of both AG and UAG, the aim of the present study was to investigate the therapeutic potential of ghrelin isoforms in diabetes-associated vascular disease and related mechanisms. Herein, we demonstrate that UAG, but not AG, systemic administration protects diabetic EPCs from senescence and restores their vasculogenic potential by regulating the small GTPase Rac1 activity. Finally, we show that UAG, unlike AG, rescues defective EPC mobilization in individuals with T2D, while having no effect in healthy subjects.

**RESEARCH DESIGN AND METHODS**

**Patients and Controls** — Blood was recovered from 14 individuals with T2D who arrived in our patient clinic (sex, M/F 8/6; HbA1c, 8 ± 1.2%; age-years, 55.8 ± 9.46; BMI, 28.1 ± 3.22; creatinine, 1.06 ± 0.20 mg/dl; waist circumference (cm), 98.8 ± 8.02; total cholesterol, 198 ± 30 mM; HDL cholesterol, 48 ± 12.98 mM; LDL cholesterol, 115 ± 38.94 mM; Triglycerides, 159.5 ± 55.23; fasting glucose 125 ± 18.03 mg/dl; no retinopathy, hypertension in 3 patients, blood pressure 142/89 mmHg; Chol/apoB, 1.3 ± 0.3). All were treated only with diet. No medications were used. Twelve blood donors were used as controls (sex, M/F 6/6; age-years, 47.8 ± 4.85; BMI 21.25 ± 8.07; creatinine, 0.90 ± 0.083 mg/dl; total cholesterol, 164.03 ± 9.68 mM; HDL cholesterol, 50 ± 8.4 mM; LDL cholesterol, 85.26 ± 16.72 mM Triglycerides, 131.14 ± 25.03; no retinopathy, no hypertension, blood pressure 142/89 mmHg; Chol/apoB, 1.3 ± 0.3). All were treated only with diet. No medications were used. Twelve blood donors were used as controls (sex, M/F 6/6; age-years, 47.8 ± 4.85; BMI 21.25 ± 8.07; creatinine, 0.90 ± 0.083 mg/dl; total cholesterol, 164.03 ± 9.68 mM; HDL cholesterol, 50 ± 8.4 mM; LDL cholesterol, 85.26 ± 16.72 mM Triglycerides, 131.14 ± 25.03; no retinopathy, no hypertension, blood pressure 142/89 mmHg; Chol/apoB, 1.3 ± 0.3). Ethical approval was obtained both from SIMT (Servizio Immunoematologia e Medicina Trasfusionale) and from the Institutional Review Board of S. Giovanni Battista Hospital, Turin, Italy. Informed consent was provided according to the Helsinki Declaration. We also declare that for the present study, we had no direct contact with human subjects.

**Testing session:**
- UAG (3.0 µg/kg/h iv. as infusion for 12 hours, from 0 to 12 hours);
- AG (1.0 µg/kg/h iv. as infusion for 12 hours, from 0 to 12 hours);
- isotonic saline (infusion from 0 to 12 hours).

All tests were performed starting at 08.30–09.00 a.m. after overnight fasting. An indwelling catheter was placed into an forearm vein for slow infusion of isotonic saline. Cells were isolated from blood samples taken at 0, 6 and 12 hours.

**Isolation, Characterization and Culture of early EPC (CACs) and late EPCs (EPCs) from Peripheral Blood Mononuclear Cells (PB-MNC)** — To isolate CACs, PB-MNC retrieved from healthy subjects (nCACs) or from individuals with T2D (dCACs) were plated on fibronectin (FN) coated dishes as described by Hill et al. (19). Briefly, the cells were cultured for 4 days in EGM-2 medium (Cambrex, Walkersville, MD, USA). To isolate EPCs (nEPCs from healthy subjects, dEPCs from individuals with T2D), PB-MNC were recovered and cultured onto collagen-1-coated dishes for 21 days in EGM-2 medium as described by Yoder et al. (26). In selected experiments, CACs or EPCs recovered from saline-infused healthy subjects were cultured with 1.2 mg/ml AGE, H2O2 (100 µM), 5 mM glucose or 25 mM glucose alone or in combination with 1µM UAG or 1µM AG. 1µM UAG or 1µM AG were also used alone. FACS analysis was used to characterize CAC and EPC surface markers (anti-CD45, anti-CD14, anti-CD34, anti-CD31, anti Tie-2, anti-KDR, anti-vWF antibodies, see online
supplemental data). eNOS expression was also evaluated.

**Diabetic and Control Mice.** — The plasma glucose and insulin determination for each group of mice (Charles River Lab, Lecco, Italy) are reported: sixteen 8 weeks-old ob/ob mice (blood glucose, 296 ± 19.6 mg/dL; insulin, 55 ± 9 ng/ml); sixteen 8 weeks-old C57BL/6J wild type mice (blood glucose, 92 ± 7.2 mg/dL; insulin, 10 ± 0.5 ng/ml). Animal procedures conformed to the Guide for Care and Use of Laboratory Resources (National Institutes of Health publication no. 93-23, revised 1985). Blood glucose was measured with a One Touch II glucose meter (Lifescane, Mountain View, CA). Serum insulin was measured with a mouse insulin radioimmunoassay kit (Linco Research immunoassay, St. Charles, MO), following the manufacturer’s instructions.

**Reagents and Antibodies** — All reagents and antibodies used are reported in the online supplemental data.

**Detection of ROS, GTP-Rac1 Loading assay, Senescence assay, Western Blot Analysis, Silencing of Endogenous p53, Akt and p47phox by small interfering RNAs (siRNA), Matrigel-plug assay, Immunohistochemistry and Immunofluorescence, Human and Mice Mobilization Assays, ELISA and RIA, Isolation and Culture of BM-derived Cells and Evaluation of MMP9 Activation, Fluocytometry Analysis, in vitro migration assays were described in detail in the online appendix supplemental section which is available at http://diabetes.diabetesjournals.org.

**Statistical Analysis** — In vitro and in vivo results are representative of at least 3 independent experiments, performed at least in triplicate. Densitometric analysis using a Bio-Rad GS 250 molecular imager was used to calculate the differences in the fold induction of protein activation or expression. Significance of differences between experimental and control values (*, **, *, †, ‡ p < 0.05, statistically significant) was calculated using analysis of variance with Newman-Keuls multicomparison test.

**RESULTS**

**UAG protects diabetic EPCs from oxidative stress by regulating Rac1.** Several lines of evidence indicate that the number and function of EPCs are impaired in diabetes (17; 19) and that these events mainly rely on Nox-mediated ROS production (15). The effect of in vivo UAG and AG administration in protecting both CACs and EPCs from oxidative damage was first evaluated. Toward this end, cells isolated from UAG- or AG-treated individuals with T2D and healthy subjects were characterized for CAC and EPC markers (Fig. S1) (18) and subjected to dichlorofluorescein diacetate (DCF-DA) fluorescence assay. The results reported in Fig. 1A and B demonstrate that UAG, but not AG, treatment (6 hours) drastically reduced ROS production in individuals with T2D. Moreover, the number of viable cells was significantly higher following UAG treatment compared to before (Fig. 1C). A protective effect of UAG on ROS production was also demonstrated in cells cultured with AGE or high glucose (Fig. S2A). Similar results were obtained when H2O2 was used (Fig. S2B). These findings, together with the observation that neither in vitro or in vivo UAG administration (Fig. S2C-D) changed AGE receptor (RAGE) expression, suggest that effector(s) downstream of RAGE is (are) the targets for protective effect of UAG. Since similar results were obtained after 12 hours of treatment (data not shown), data throughout the study relates to 6 hour UAG treatment.

Apocynin, known to affect the assembly of Nox subunits (27), was used to investigate the role of Nox in regulating ROS production. Cells recovered from saline-infused individuals with T2D were subjected to apocynin treatment. ROS production was prevented in both dCACs and dEPCs (Fig.
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1D). UAG addition could not further enhance apocynin effects (Fig. 1D), suggesting that Nox activity might be controlled by UAG. As the assembly of p47\textsuperscript{phox} and p67\textsuperscript{phox} subunits is required for Nox enzymatic activity (28), silencing of p47\textsuperscript{phox} in dCACs and dEPCs (Fig. S3A) prevented ROS production (Fig. 1E). Once more this effect could not be further enhanced by the addition of UAG (Fig. 1E). Nox2 activity is also dependent on the small GTPase Rac (28). ROS generation in response to AGE (Fig. S3B-C) was prevented in cells expressing a dominant negative RacN17 construct. We thus hypothesized that UAG interfered with Rac1 activity. Indeed, Rac1 activation was detected in cells recovered from saline-treated individuals with T2D, but not from UAG-infused patients (Fig. 1F). Similar results were obtained \textit{in vitro} by culturing cells with AGE (Fig. S3D). Hence, the modulation of Rac1 activity is a crucial step in UAG anti ROS protective effect.

Rac1 membrane localization and function rely on isoprenylation which has been correlated with AMPK-dependent HMG CoA reductase activity (29). To rule out the possibility that inhibition of Rac1 activity by UAG depends on this pathway, AMPK phosphorylation was evaluated. Neither short or long term exposure to UAG alone or in combination with AGE affected AMPK activity (Fig. S4).

\textbf{UAG prevents cell senescence and improves de-novo vessel formation.} p53, p21 and pRb are major regulators of cell senescence (30). The above results prompted us to evaluate whether the increase in ROS production, generally considered as an upstream signal, translates into an accelerated onset of senescence and whether UAG could rescue this effect. Since both senescence and ROS generation (Fig. 2A and Fig. 2B) were prevented by silencing p53 (Fig. S5A), we investigated the \textit{in vivo} effect of UAG on p53 expression. Accordingly, UAG treatment was able to prevent p53 accumulation, p21 expression and Rb phosphorylation (Fig. 2C) and to reduce the number of SA-β-gal positive dCACs and dEPCs (Fig. 2D) in UAG-challenged patients. Similar results were obtained in AGE-treated cells (Fig. S5B-C).

To assess whether the protective effect of UAG also resulted in an enhancement of dCAC and dEPC vasculogenic capability, \textit{de-novo} vessel formation was analyzed in SCID mice injected with cells recovered from UAG-treated individuals with T2D. 15 days after injection, plugs were recovered and analyzed by immunohistochemistry. As shown in Fig. 3A and 3B, the number of functional vessels formed by cells recovered from UAG-treated patients was significantly increased with respect to those from saline-treated patients. The origin of neo-vessels from host vasculogenic cells was excluded since the majority of vessels were lined by human HLA Class I positive cells (Fig. S6) (31). Thus, these data provide evidence that UAG restores dCAC and dEPC vasculogenic activity.

\textbf{UAG improves EPC mobilization.} Defective EPC and CAC mobilization has been reported in diabetes (19; 20). To further investigate the potential therapeutic effect of UAG, CACs and EPCs were recovered from 10 normal healthy subjects (N) and 10 individuals with T2D (D), characterized and counted. UAG treatment led to an increase in the number of recovered cells in individuals with T2D compared to that of healthy subjects, and no effect of UAG treatment was detected in healthy subjects (Fig. 4A). In contrast, no differences between AG- or saline-treated healthy and individuals with T2D were observed (Fig. 4A). Additionally, as shown in Fig. 4B the percentage of circulating senescent dCACs and dEPCs decreased upon UAG treatment.

Circulating Stromal Derived Factor-1 (SDF-1) (32) and Vascular Endothelial Growth Factor (VEGF) (33) strictly control progenitor cell mobilization under stress...
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UAG mobilizes EPC by rescuing eNOS activity. For validation and characterization of the molecular mechanisms regulating BM mobilization, a mouse model of type 2 diabetes (ob/ob mice), was used. After treatment with saline, UAG or AG, recovered cells were subjected to FACS analysis for surface markers to confirm EPC identity (data not shown). UAG treatment induced a strong increase of recovered EPCs only in ob/ob mice (Fig. 4C). Finally, the number of senescent cells was significantly lower in UAG-treated ob/ob mice as compared to untreated- or AG-treated animals (Fig. 4D), reproducing our findings in human subjects. As an impairment of eNOS phosphorylation contributes to defective EPC mobilization in diabetic setting (35), we investigated whether UAG modulated eNOS activity and the activation of its regulatory protein, Akt (36), in BM-stromal cells. The stromal origin of the eNOS expressing cells was confirmed by the presence of the membrane bound Kit ligand (mbKitL) (Fig. 5A). In ob/ob mice, UAG treatment restored both eNOS and Akt phosphorylation (Fig. 5A). Consistent with the pivotal role of a local activation of MMP9 in promoting progenitor cell mobilization (22), gelatin-zymography revealed that MMP9 gelatinolytic activity was induced by UAG (Fig. 5B). The role of MMP9 activation and sKitL release in controlling this event was further confirmed by functional studies using anti-MMP9 and anti-KitL neutralizing antibodies (Fig 5C). Accordingly, in parallel with effects on MMP9 activation, expression of the mbKitL was decreased in ob/ob mice subjected to UAG treatment (Fig. 5A). Although we cannot rule out the possibility that a paracrine effect of UAG occurs in vivo, herein we have shown that in vitro UAG treatment for 40 minutes elicited Akt and eNOS phosphorylation in stromal cells obtained from ob/ob-derived total BM pools (Fig. 5D). In agreement with the results measuring EPC mobilization, AG failed to induce Akt and eNOS phosphorylation (Fig. 5D). The finding that UAG failed to induce eNOS phosphorylation after knocking down Akt (Fig. 5E) or inhibiting its activation (Akt-inhibitor) (Fig. 5D) indicates that Akt is a key modulator of the UAG effect. The above data were validated by the lack of effect of UAG treatment in NOS3+/− mice (23) (Fig. S8).

CACs express specific UAG binding sites. Based on our collective data set, we further investigated whether the biological response of CAC to UAG was mediated by specific binding sites localized to the plasma membrane. To this end, double immunofluorescence experiments, using the UAG analogue 488-UAG (100 nM) to label putative binding sites and anti-PE-CD45 antibody as a membrane marker, were carried out at 4°C. As shown in Fig. 6A, at 4°C 488-UAG-binding sites co-localized with CD45, indicating a plasma membrane localization. In agreement with the functional data, unlabelled UAG (1 µM) displaced the fluorescent signal from the cell surface while AG (1 µM) did not (Fig. 6A). Additionally, we also monitored receptor activation at 25˚C. As shown in Fig. 6B, after 20 minutes stimulation, receptor clusters undergoing internalization were visualized as labelled cytoplasmic vesicles. Moreover, increasing concentrations of unlabelled UAG specifically displaced the fluorescent ligand from both the plasma membrane and endocytic vesicles (Fig. 6C and Fig. S9). Similar results were obtained using EPCs (data not shown).

DISCUSSION

The present data first demonstrate that i.) UAG, unlike AG, reverts diabetes-induced...
EPC damage, by inhibiting activation of the Nox regulatory protein Rac1; ii.) as a consequence, UAG protects diabetic EPC from senescence and improves their vasculogenic capability; iii.) again, only UAG rescues EPC mobilization under diabetic conditions by restoring eNOS phosphorylation; and iv.) specific UAG binding sites mediate its effects.

UAG is the most abundant circulating form of ghrelin (3) and plays a positive role on glucose metabolism. In contrast, basic and clinical studies have proposed AG as a diabetogenic hormone (8; 37). Indeed, clinical conditions of insulin resistance are associated with an alteration in the circulating ghrelin profile with relative AG excess with respect to UAG (12). Thus, it is tempting to speculate that an altered UAG/AG ratio might contribute both to metabolic changes and to diabetes-associated complications.

Among diabetes-associated complications, abnormal vascular remodelling is believed to play a major role in accelerating vascular disease (38). Historically, it has been assumed that new blood vessels originate from sprouting cells and co-opting of neighbouring pre-existing vessels. However, both physiological and pathological angiogenesis are also supported by mobilization and recruitment of other cell types, including the BM-derived cells, such as EPC (16;18; 39; 40). Interestingly, alterations in the number and function of these cells correlate with the risk factor profile (19; 20). Overproduction of ROS in these pathological settings seems to contribute to impaired vascular regenerative processes (41). The plasma membrane Nox is recognized as one of the major regulators of ROS generation (15; 28). Activation of the enzyme can occur via many upstream signalling pathways converging on phosphorylation of p47^phox and activation of Rac1 leading to the oxidase assembly (28). Our study shows that UAG can protect diabetic EPC from oxidative stress by affecting the Nox regulatory protein Rac1.

The accelerated onset of senescence contributes to the impaired EPC bioavailability in patients with diabetes (17). The tumour suppressor p53 is a transcription factor involved in DNA damage mechanisms and is recognized as a negative regulator of cell proliferation in human atherosclerotic and restenosis lesions (42). Moreover, the p53-mediated pathway contributes to EPC senescence-like growth arrest in diabetes (43). Accordingly, the present study shows that silencing p53 in EPCs isolated from individuals with T2D or cultured with AGE prevents both ROS production and senescence. The finding that both in vitro and in vivo exposure to UAG negatively modulates p53 accumulation, identifies the p53-mediated signal as the primary mechanism through which UAG protects EPCs from senescence. We also found that, by preventing oxidative stress, UAG improves de-novo vessel formation. The efficacy of cell therapy certainly depends on the number, functional capability and successful retention of cells in the site of action. Thus, our data strongly suggest that naturally occurring UAG induces improvement of EPC function and survival which might translate into a more efficient response to vascular dysfunction.

Senescence is also associated with impaired mobilization of BM-derived cells (17). The molecular interactions between stem cells and BM stromal cells, and the molecular mechanisms controlling their mobilization in BM microenvironment, are poorly understood (44). Considerable interest has arisen on agents able to mobilize and augment progenitor cell delivery to sites of vascular injury to enhance revascularization. Among these, SDF-1 and VEGF are recognized as primary regulators of BM cell mobilization during stress conditions (22; 45). In addition, under physiological stresses, the
activation of matrix proteases within the BM microenvironment results in the release of sKitL that unables endothelial and hematopoietic progenitor cells to transit from the quiescent to the proliferative niche and facilitates their mobilization into the circulation (22). The delivery of progenitor cells to sites of neovascularization also relies on functional eNOS activity (23). Indeed, in pathological settings associated with blunted eNOS activity and reduced systemic NO bioavailability, defective EPC mobilization and impaired vascular regenerative processes occur (24; 25). eNOS activation through Akt has been reported for AG acting on the Gq-coupled GHS-R1a in cultured endothelial cells (46). We herein demonstrate that UAG can restore eNOS activity via Akt-mediated phosphorylation in a pathological setting characterized by impaired eNOS phosphorylation. This was particularly true in BM-stromal cells from diabetic mice. Such an event was found crucial to EPC BM-mobilization, as UAG had no effect in eNOS knockout mice. Gu et al. (47) identified MMP9 as a major target of NO. We also showed that eNOS phosphorylation, occurring in response to UAG, is associated with MMP9 activation and possibly with the release of sKitL, as suggested by the reduced expression of the mbKitL on stromal cells recovered from UAG-treated mice and by functional studies. Although we cannot exclude that UAG may act in a paracrine manner by locally inducing the release of VEGF from BM stromal cells, we demonstrate that short term treatment with UAG, but not AG, of BM cells in vitro leads to Akt and eNOS phosphorylation and that, in vivo, these events are associated with MMP9 activation and EPC mobilization. Furthermore, UAG, unlike AG, strongly induced EPC mobilization in individuals with T2D, but not in non-diabetic subjects. Notably, no change in serum concentrations of primary mobilization factors was detected after UAG or AG systemic administration.

In addition, our results shed light on the earliest molecular events leading to UAG-but not AG-mediated physiological regulation of EPC bioavailability. Indeed, we showed that EPCs possess specific UAG binding sites, which are not recognized by AG. In addition to GHS-R1a, which is the AG specific receptor (3), other ghrelin receptors subtypes exist, whose molecular identities have not yet been characterized, but which recognize both AG and UAG (3). Thus, our data provide the first evidence of the existence of UAG-specific binding sites and of their relevance in human-derived EPCs, that could represent a novel target for pharmacological modulation.

Preclinical and clinical studies generally support the therapeutic potential of autologous EPC in the treatment of cardiovascular diseases, such as tissue ischemia and myocardial infarction (48). However, EPC mobilization may also accelerate atherosclerotic plaque progression (49) and induce tumor (50) or retina neovascularization (51) in individuals with T2D. Nonetheless, therapies with statins, the main EPC mobilization mediators (52), revealed no concerns in terms of neovascularization. We now have reasons to believe that the significant EPC mobilization potential of UAG and the lack of its effects on serum levels of SDF-1 and VEGF, may be exploited for clinical treatment of diabetes- and atherosclerosis-induced vascular impairment.

In the presence of cardiovascular risk factors such as diabetes, EPC availability is reduced, restricting the possibility to treat patients in need with directed/cell-based therapies. Besides displaying a positive influence on β-cell viability and glucose homeostasis (3), UAG mobilizes EPCs, protects EPCs from oxidative stress and from senescence and increases de novo vessel formation (see model in Fig. 7). This suggests
that UAG related peptides or UAG receptor-specific agonists might be further developed into lead compounds from the perspective of a novel pharmacological intervention to ameliorate both metabolic control and impaired vascular growth in individuals with T2D where AG has failed.

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FIGURE LEGENDS

Figure 1. UAG, by preventing Rac1 activation, protects CACs and EPCs from ROS production. (A-B) DCF-DA assay was performed on CACs or EPCs, recovered from healthy subjects (nCACs, nEPCs) or from diabetic patients (dCACs, dEPCs) treated with saline, UAG or AG (white area: no treatment; dark grey area: UAG-infused; light grey area: AG-infused) (C) CACs and EPCs isolated from healthy donors (N) and from diabetic patients (D) treated as above were cultured as described in Research Design and Methods. Trypan blue-excluded viable cells were counted. (*, § p < 0.05, healthy donors vs diabetic groups; # p < 0.05, saline vs UAG infusion). (D) ROS production, in response to apocynin alone or in combination with UAG, was evaluated on dCACs and dEPCs isolated from saline-treated patients (white area: no treatment; dark grey area: apocynin; light grey area: apocynin + UAG). (E) ROS production was evaluated on dCACs and dEPCs isolated from saline-infused patients and transfected with the scramble-sequence or with p47phox siRNA. Cells were treated or not with UAG (white area: scramble; dark grey area: p47phox siRNA; light grey area: p47phox siRNA + UAG). (F) dCACs and dEPCs retrieved from saline- or UAG-infused patients were analyzed for Rac1 activation. Cell extracts were either pulled down with GST-PAK or directly subjected to SDS-PAGE. Eluates or extracts were immunoblotted with an anti-Rac1 antibody. GTP-Rac1 and total Rac1 are indicated. IL-3-treated endothelial cells were used as positive control (+). CACs and EPCs were recovered following a 6 hour infusion with saline or UAG as AG indicated.

Figure 2. UAG prevents dCAC and dEPC senescence. (A) Senescence was evaluated on dCACs and dEPCs recovered from saline-infused patients transfected with pSUPER retro containing p53 siRNA or the scrambled sequence as control, and expressed as the percentage of SA-β-gal-positive cells (*p < 0.05, scramble vs p53 siRNA). (B) ROS production was evaluated on dCACs and dEPCs recovered from saline-infused patients transfected with pSUPER retro containing p53 siRNA or the scrambled sequence (white area: scramble; grey area: p53 siRNA). (C) dCACs and dEPCs retrieved from saline- or UAG-infused patients were lysed and analyzed for p53, p21 and pRb expression by western blotting. Ox-LDL-treated endothelial cells were used as positive control (+). (D) Senescence was evaluated on dCACs and dEPCs recovered from saline- or UAG-infused patients and expressed as the percentage of SA-β-gal-positive cells (*p < 0.05, saline vs UAG infusion).
Figure 3. UAG increases *de-novo* vessel formation. (A) Immunohistochemistry of representative functional vessels formed by labelled dCACs and dEPCs, recovered from saline-(left) or from UAG-infused (right) diabetic patients. Black arrows indicate labelled cells (40X magnification). (B) Quantification of newly-formed vessels was expressed as percentage ± SD of the vessel area to the total Matrigel area (*p < 0.05, saline-infused vs UAG-infused).

Figure 4. UAG induces CAC and EPC mobilization. (A) Percentage of CACs and EPCs recovered after 6 hours of saline, UAG or AG treatment: (N) healthy donors and (D) diabetic patients, (*p < 0.05, diabetic patients vs healthy donors, *# p < 0.05 saline vs UAG infusion). (B) Senescence was evaluated on CACs and EPCs recovered as described in (A) and expressed as the percentage of SA-β-gal-positive cells (*p < 0.05, diabetic patients vs healthy donors, # p < 0.05 saline vs UAG infusion). (C) Percentage of EPCs recovered from wild type or ob/ob mice infused for 12 hours with saline, UAG or AG (*p < 0.05 WT UAG-infused vs ob/ob UAG-infused mice). (D) Senescence was evaluated on murine EPCs treated as in described in (C) and expressed as the percentage of SA-β-gal-positive cells (*p < 0.05, WT vs ob/ob mice, # p < 0.05 saline vs UAG infusion). All data are the mean ± SD obtained by three individual investigators.

Figure 5. UAG restores eNOS phosphorylation. (A) Adherent cells (from total BM populations) obtained from saline-, UAG or AG-treated WT or ob/ob mice were lysed and analyzed by western blotting using the indicated antibodies. The blots are representative of two WT (1-2) or three ob/ob (3-5) samples. IL-3-treated endothelial cells were used as positive controls. (B) Representative zymogram of MMP9 gelatinolytic activity on serum recovered from saline- or UAG-treated mice. As positive control, human serum was used (+). (C) Migration assays performed on BM-cells using sera recovered from saline- or UAG-treated ob/ob mice. MMP9 or sKitL neutralizing antibodies were added where indicated (*p < 0.05, saline vs UAG, # UAG vs UAG+ anti-MMP9 and UAG+ anti-sKitL). (D) Adherent cells obtained from ob/ob total BM pools were treated as indicated for 40 minutes and processed for western blotting using the indicated antibodies. (E) Adherent cells from ob/ob total BM pools were depleted (siRNA Akt) or not (scrambled) of Akt, subjected to UAG treatment and analyzed by western blotting using the indicated antibodies.

Figure 6. Visualization of UAG binding sites on CACs by 488-UAG. (A-C) Living cells were incubated in absence (CTRL) or in presence of 100 nM 488-UAG with or without the indicated ligands. The putative receptor is stained in green (488-UAG), the plasma membrane in red (PE-CD45) and the nucleus in blue (DAPI). (A) Visualization of 488-UAG binding site distribution on CACs plasma membrane. CAC incubation with ligands was carried out at 4°C for 4 hours. 488-UAG was displaced by 1µM UAG but not by 1µM AG (40X magnification). (B-C) Visualization of 488-UAG binding distribution on CACs upon incubation with the indicated ligands for 20 minutes at 25°C. (B) Representative photomicrograph showing the 488-UAG-labelled cell surface binding sites internalized into endocytotic vesicles (20X magnification). (C) 488-UAG-binding and internalization were competed by increasing concentrations of the unlabelled UAG.

Figure 7. Schematic model for UAG impact on EPC physiology. The resident BM pool of early and late EPCs were mobilized into circulation following UAG-mediated Akt activation in stromal cells, that leads to eNOS phosphorylation and MMP9 activation. On turn, MMP9 activation switched on the release of sKitL, determinant for EPCs exit into the blood stream. Additionally, UAG treatment restores diabetic EPC viability and increases their vasculogenic capability by preventing ROS generation and its downstream signalling pathway (p53 accumulation, p21 expression, Rb phosphorylation). This effect results from UAG-mediated
inhibition of Rac1 activation, a pre-requisite for NADPH oxidase assembly. BM (bone marrow compartment); PC (peripheral compartment).

Figure 1
Figure 2

UAG improves vascular repair
Figure 3

A  saline       UAG

\[ \text{dCACs} \]
\[ \text{dEPCs} \]

B

\[ \text{mean area (％) } \]

\begin{align*}
\text{dCACs} & \quad \text{saline} & \text{UAG} \\
\text{dEPCs} & \\
\end{align*}

\[ \star \]

UAG improves vascular repair
Figure 4
Figure 5

A

B

C

D

E

UAG improves vascular repair
Figure 6

A

CTRL  
488-UAG  
AG 1 µM  
UAG 1 µM

B

CTRL  
488-UAG

C

CTRL  
488-UAG  
10 nM  
100 nM  
1 µM  
UAG

UAG improves vascular repair
UAG improves vascular repair

Figure 7

BM

BM stromal cell
BM progenitors
mb-KiIL
C-Kit

UAG

PC

AGE
RAGE
NADPHox
Rac1
ROS

senescent
not vasculogenic
EPCs

EPCs

UAG

NOS

NADPHox

viable
vasculogenic
EPCs

type